Food Research

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WHY SHOULD THE VITAMIN C CONTENT OF FRUIT AND VEGETABLE JUICES BE STANDARDIZED?

Because nutrition reports disclose that—

- **Vitamin C deficiencies exist throughout the country***

Proof of deficiencies among adults and children. You should know these facts!* Diet surveys from Maine and Vermont, New York, Illinois, New Mexico, Oregon, West Virginia, and Texas show a definite pattern of inadequate vitamin C intake. These studies covered grade school and high school children, college men and women.

Spongy, swollen gums symptomatic of scurvy.

From these studies* of dietary habits and physical conditions there is considerable proof that many of the children and adults covered by the surveys have actual, physical symptoms of vitamin C deficiency.

Infantile scurvy. Scurvy* occurs, even today, among infants who are artificially fed. Fortunately the disease may be cured by the administration of vitamin C. How much more sensible it is to prevent it by supplying these bottle-fed infants with the daily allowance of 30 milligrams of vitamin C as recommended by the Food and Nutrition Board of the National Research Council for children under one year of age.

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- **Scurvy has not disappeared from the United States***

Typical position of legs in infantile scurvy.

What can be done? Because of the increasing evidence that American diets are deficient in vitamin C, leading nutrition experts believe that processed fruit and vegetable juices, on which the public is so reliant for its supply of this necessary vitamin, should contain the "Vital Ingredient."

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*Statements are based on documented reports. References on request.

Illustrations after photographs in The Vitamins in Medicine by Bicknell and Prescott, Third Edition, published by Grune & Stratton, Inc., New York.

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ANALYSIS OF SCORES FOR BITTERNESS OF ORANGE JUICE

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(Manuscript received June 6, 1955)

A major problem in canning orange juice is the development of a bitter taste due principally to the presence of limonin. This bitterness is associated with factors such as rootstock, scion, and picking maturity. Hence, experiments over a period of some years have been conducted to compare juices from fruit grown on various rootstock-scion combinations. A panel of tasters using a rating scale for bitterness has made the comparisons. Over a considerable period the panel used a rating scale of 0 to 4 and, on the basis of their performance, the best tasters were selected. The tasters became trained and experienced in the use of this scale.

From the restricted nature of the distribution of the discrete variate values, it is clear that the assumptions of normality and constancy of error variance require justification before statistical treatment by analysis of variance can be taken to lead to valid conclusions. Hopkins *(8)* has stated that food technologists are mainly concerned with the central part of the scale range, and it has been found in at least some instances that the variance may, for practical purposes, be regarded as independent of mean score. It is obvious that when the end points of the scale become operative restrictions to scoring, the above cannot be assumed to obtain, and a mean-variance association might be expected. The juices in the experiments often included some not markedly bitter and data from such tests presented difficulties in statistical analysis. As the experiments progressed and the juices became less bitter through successful elimination of various causes, these difficulties increased. Hence, it was desirable to study this problem so that valid analyses of bitterness scores which tended to accumulate at the lower end of the rating scale could be made.

METHODS

Scale. The difficulty due to discreteness of the scale was lessened by using intermediate half points, and the tasters were trained to use the modified scale (Table 1).

Obtaining sufficient data to give reliable estimates of means and variances entailed extensive repetition of tests; this is unusual and presented difficulties.

Elimination of after-taste. After tasting certain materials some after-taste persists in the mouth; this was confirmed by Cartwright and Nanz *(4)* for spices and Boggs and Ward *(3)* for sulphited foods. If a number of such materials are to be rated at each session, after-taste prevents the proper rating of samples. From the experience of the tasters it was expected that the bitter principle of orange juice would come into this category. One way to minimise this effect would be to present the samples in increasing order of bitterness as was done for the comparison of texture of peas by Boggs *(2)*. This would prevent the presentation of samples in random order and make it easy for the tasters to recognise them from one session to another. The only satisfactory solution is to use some substance which will eliminate the after-taste. Physiological saline and bread were tried.

[1]Division of Mathematical Statistics, C.S.I.R.O.

TABLE 1
Scale of scores for bitterness

Score	Description
0	No bitterness
1	Slight bitterness
2	Moderate bitterness
3	Pronounced bitterness
4	Very pronounced bitterness

Intermediate half points are regular scale points

Tests were arranged in the design of a Latin square, balanced for residual effects of the first order as developed by Williams *(12)*, there being as many replications of squares as the number of tasters allowed. Analysis of the untransformed scores showed that there was an effect of after-taste; it was not evident if the tasters were delayed between samples and this accords with the value of pauses as commonly recommended in the literature. Analysis showed that bread was effective in eliminating after-taste. The saline seemed effective too but it was ejected by the tasters and because of the inconvenience and unpleasantness of this, its use was regarded as generally unfavorable. Hence, bread was used throughout the experiment and there was complete freedom for random presentation of samples.

From time to time during the course of the experiment the individual randomizations for tasters were arranged for the design used above; analyses showed that the effect of after-taste had been eliminated. The mean scores of the juices ranged from 0 to 2½ approximately and the conditions required by the underlying assumptions are probably not fully met. In spite of this, there seems little doubt that the samples were properly and independently scored.

Study of mean-variance relation and experimental procedure. The 6 samples of juice were selected to cover the scale from 0 to 2½ by half points approximately. It was considered that no more than 4 samples should be scored at any one session and hence the work was arranged in three tests. The 6 juices are designated A, B, C, D, E, and F (increasing order of bitterness) and the tests were constituted as follows:

Test	Juices
1	A, B, C, and D
2	A, C, and E
3	B, D, and F

Each test was repeated until 12 replicates of scores were obtained. Successive sessions used tests at random so that it would have been difficult for tasters to guess the design. The experiment was safeguarded further by the occasional use of dummy sessions. Because juices A, C, E, and F were from Washington Navels and B and D from Valencia oranges, there were some colour differences, the first group being paler. Any influence of these differences was obviated by using orange-coloured glasses. Slight differences in acidity and sweetness should not have influenced the results under the conditions of the experiment. The score for each sample was recorded as tasted. The portion tasted was about one ounce. Samples were served at room temperature as representing normal conditions. Complete data were obtained from 31 tasters.

RESULTS

Distribution of scores. The frequency-distributions of scores were J-shaped for A, unimodal for B to D with positive skewness decreasing until, for E and F, they were apparently symmetrical.

Influence of taste threshold. The threshold of bitterness for a substance may be defined as the minimum concentration causing the sensation of bitterness. This concentration has been found to vary between individuals and with time for any individual; Salmon and Blakeslee *(11)* confirmed this. A zero score for an individual must be interpreted to mean that the concentration of the bitter principle was below the

threshold for that taster at that time. Further, any non-zero score to mean that the concentration was above the threshold. Hence, if we consider a mean bitterness threshold for tasters and times, and ignore sampling variation for the juice, the distribution of scores for juice A shows that the concentration of limonin was below the mean threshold. Similarly, it is clear that the concentration of limonin in juice B was above the mean threshold. Hence, the limonin concentration for juices A and B would give some estimate of this as yet unknown quantity; data for juices C and D would also be of assistance. Unfortunately, the chemical work necessary to obtain this information is involved and difficult.

The experiment provided clear evidence of the difference in threshold for tasters. χ^2 was calculated for juice A for scores classified as zeros and non-zeros; the minimum cell expectation was close to 5, but, following Fisher *(6)*, the test is approximately valid. The χ^2 values were 158 (Test 1) and 152 (Test 2) on 30 degrees of freedom (Tables give $\chi^2 = 59.7$ for $P = 0.001$).

Statistical analysis: Application of transformation. As far as the statistical analysis of the scores is concerned, the scale points can be regarded as discrete measures of a continuous variate, viz., limonin concentration. This discrete scale is displaced to and fro for tasters and times because of the change of threshold. The distributions of the scores leads to the conclusion that, for juices A and B in particular, the discrete scale was unable to follow the continuous scale below a certain point, viz., the threshold concentration, so that the actual frequency distribution might be described as a type of truncation. This effect was not pronounced for juices C and D but quite marked for A and B so that analysis of variance of the untransformed scores would be adversely affected. If we denote the scores by x_{ijk} where

$i = 1, 2, \text{----}, 31$ for tasters,
$j = 1, 2, \text{----}, 4$ (3) for juices in test 1 (tests 2 and 3),
$k = 1, 2, \text{----}, 12$ for sessions,

then, the mean score for a juice is $\bar{x}_{.j.}$. If there was an association of variance with $\bar{x}_{.j.}$ it could reasonably be expected to apply to both sessions and tasters as sources of variation. The estimates $S\,(x_{ijk} - \bar{x}_{.j.})^2/n$ of this total variance have been calculated for each juice in each test; n denotes the number of degrees of freedom, i.e. 371. These estimates are plotted in (a), (b), and (c) of Figure 1 against their respective means $\bar{x}_{.j.}$. Parabolas of the form $y = \lambda \bar{x}_{.j.}\,(4 - \bar{x}_{.j.})$ have been fitted to the points for each test separately; these curves suggest that this equation accounts for the mean-variance relation very satisfactorily.

This relation was studied by Hopkins *(8)* who also fitted such curves: The general nature of the relation was suggested by Cochran *(5)*. The constant λ has been evaluated

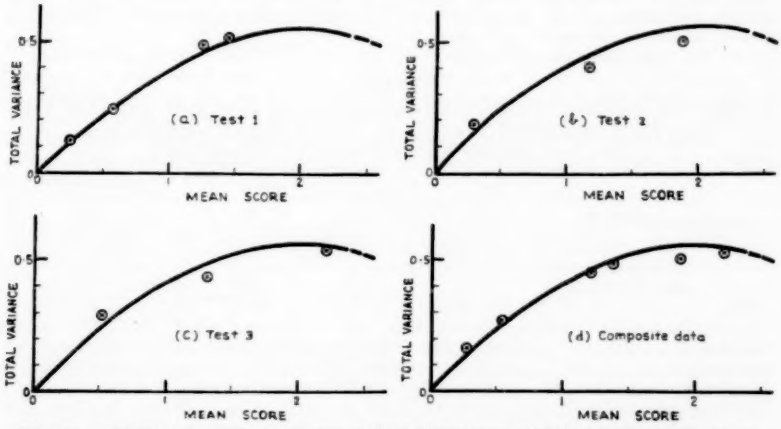

Figure 1. Graphs of juice mean score and total variance with fitted parabolas shown.

to minimise the sum of weighted squares of deviations from the curve. The weights were taken, in the first instance, from the expression $2\sigma^4/n$ for the variance of an estimate of the variance σ^2 on n degrees of freedom, the estimate s^2 being used as an approximate value of σ^2. In the second and any further steps of the iteration weights have been obtained from the predicted variances.

The only variation between mean scores for the same juices in different tests not accounted for by error of scoring and sampling variation for the juice, is due to variation in scoring level between sessions; this would be small when averaged over 12 sessions. Hence the overall estimates of mean and total variance for juices A, B, C, and D have been calculated and grouped with the values for E and F from tests 2 and 3 to obtain composite data. These values are plotted in (d) of Figure 1 which shows the best fitting parabola; it appears that it fits the points satisfactorily. The closeness of fit of these curves was tested by calculating $\chi^2 = S\,(Y - y)^2/\sigma^2$, where y is the observed value of total variance, Y the predicted value from $Y = \lambda\,\bar{x}_{.j.}\,(4 - \bar{x}_{.j.})$, and σ^2 the variance of y obtained from $2\,\sigma^4/n$ by substituting Y for σ^2. The values of χ^2 were:

Test	χ^2	Degrees of freedom	Probability
1	2.22	3	$0.70 > P > 0.50$
2	13.54	2	$0.01 > P > 0.001$
3	5.96	2	$0.10 > P > 0.05$
Composite data	7.79	5	$0.20 > P > 0.10$

Hence, it appears that this relationship is fairly satisfactory and might be used to obtain a transformation.

By using the approximate value for the variance of a function of a variate given by Bartlett *(1)*, it follows that the transform with constant arbitrary variance k, is given by the function

$$f(x) = \sqrt{k}\int \frac{dx}{\sqrt{\lambda\,x\,(4-x)}} = 2\sqrt{\frac{k}{\lambda}}\,\text{arc sin}\,\tfrac{1}{2}\sqrt{x}\ \text{approximately},$$

where the variance of the variate x is related to its mean by the relationship variance $= \lambda\,\bar{x}\,(4 - \bar{x})$. This transformation is not appropriate to the original scores, but might be applied to means of suitable groups of scores of which there are replicates. In an experiment to compare juices from certain rootstocks there would properly be samples from replicate trees of these rootstocks. In the present experiment these replicates were dummy and corresponded to the samples compared at separate sessions. Hence the transformation was applied to the means $\bar{x}_{.jk}$, the panel mean scores for individual samples and sessions. The variance ratios F of the resulting analyses of variance for Tests 1, 2, and 3 are shown in Table 2.

TABLE 2

F values from analyses of variance: 'transformed' denotes those from analysis of panel mean scores transformed to arc sin $\frac{1}{2}\sqrt{\bar{x}_{.jk}}$ (degrees), 'untransformed' those from analysis of panel mean scores untransformed

Source of variation	(1) Test 1			(2) Test 2			(3) Test 3		
	D f	F		D f	F		D f	F	
		Transformed	Untransformed		Transformed	Untransformed		Transformed	Untransformed
Juices	3	491.50***	496.32***	2	817.20***	603.52***	2	1130.58***	1170.62***
Sessions	11	2.09*	1.89	11	2.56*	2.05	11	3.84**	3.54**
Error	33			22			22		
Total	47			35			35		

D f denotes degrees of freedom. One, two, and three asterisks denote significance at $P = 0.05$, 0.01, and 0.001 probability levels, respectively; F values not so marked are not significant at $P = 0.05$.

The magnitude of experimental error is of interest when compared with the expected error calculated from the transformation and the estimate of λ from the best fitting parabola. The theoretical errors calculated from the values of λ for the parabolas fitted to total variances, taking into account that panel means were transformed, are $(90^2\lambda)/(31\pi^2)$. Parabolas were also fitted for the mean-variance relationship using variances of panel means $\bar{x}_{jk}$ estimated on 11 degrees of freedom in which case the the theoretical variance is $(90^2\lambda)/\pi^2$. The resulting figures are:

	Test 1	Test 2	Test 3
Theoretical variance $(90^2\lambda)/(31\pi^2)$	3.604	3.668	3.642
Theoretical variance $(90^2\lambda)/\pi^2$	3.291	4.266	3.787
Actual variance	2.642	2.833	1.951

The theoretical errors are greater than the experimental but it was found that the two were not significantly different at $P = 0.05$ in any test. Sessions were ignored as blocks and within-juice-between-samples estimates of variance on 11 degrees of freedom calculated for the transformed data. All groups of estimates satisfied Bartlett's test for homogeneity, the χ^2 values and degrees of freedom being 3.18 (3), 2.74 (2), and 0.67(2) for Tests 1, 2, and 3, respectively. The juice means, standard errors, etc. are given in Table 3.

TABLE 3

Juice mean scores following analysis of transformed panel means; those for analysis of untransformed panel means are shown in parentheses when different

	(1) Test 1		(2) Test 2		(3) Test 3	
	Angle(°)	Score	Angle(°)	Score	Angle(°)	Score
Juice						
A	14.42	0.25	15.86	0.30	.....	.....
B	22.14	0.57	.....	.....	21.06	0.52
C	33.81	1.24	32.81	1.17(1.18)	.....	.....
D	36.83	1.44	.....	.....	35.10	1.32
E	.....	.....	43.40	1.89	.....	.....
F	.....	.....	.....	.....	48.17	2.22
S.E. of mean	±0.47	±0.025	±0.49	±0.032	±0.40	±0.025
Degrees of freedom	33	33	22	22	22	22
Least significant differences						
P = 0.05	1.35	0.072	1.42	0.095	1.18	0.073
P = 0.01	1.82	0.097	1.94	0.129	1.61	0.099
P = 0.001	2.40	0.128	2.60	0.174	2.16	0.133

Correlation of mean and variance. The above statistical treatment dealt with panel mean scores and transformed them. Taking into account the obvious non-normality of certain distributions and the fact that there were 31 tasters, the panel means might be expected to be approximately normally distributed. If, in addition, their variances were not associated with their means, there would be no need for the transformation. The within-juice-between-samples estimates of variance of these panel means were tested for homogeneity by Bartlett's test. The results were:

Test	χ^2	Degrees of freedom	Probability
1	4.63	3	$0.30 > P > 0.20$
2	12.32	2	$0.01 > P > 0.001$
3	0.99	2	$0.70 > P > 0.50$

These results did not specifically deal with any trend. For a trend near enough to linear the product moment correlation coefficient is a sufficient test. Table VI of Fisher and Yates' Statistical Tables *(7)* shows that, because the degrees of freedom in the separate tests are small, it is unlikely that any trend could be established in the tests individually. The estimates of mean and variance can, with little error, be put in a single group on the same basis. These estimates are shown in Table 4 and show a closely linear rela-

tionship. They gave a correlation coefficient of $r = 0.677$ on $n = 8$ degrees of freedom ($P < 0.05$). The corresponding estimates of variance on the transform are also shown; their correlation coefficient with the means is $r = 0.090$ on $n = 8$ degrees of freedom ($P = 0.8$ approx.).

Statistical analysis of untransformed panel means. For purposes of comparison the untransformed panel means were analysed. The F values are shown in Table 2; the juice mean scores corresponding, together with standard errors, etc., are set out in Table 3.

Influence of panel size. It must be remembered that in routine tasting tests there might be only about eight panel members, whereas in the above investigation 31 were used. Hence the data for eight (and four) efficient tasters [the first eight (four) of Table 8 of Appendix I] were analysed as detailed above. The salient points from these analyses for four tasters are presented in Table 5 using the notations (and degrees of freedom) of Tables 2 and 3.

TABLE 4

Juice mean scores and variances

Test	Juice	Mean score	Estimated variance between sessions within juices	
			From panel means untransformed	From panel means transformed
1	A	0.25	0.003,62	3.30
2	A	0.30	0.003,09	2.35
3	B	0.52	0.009,75	4.44
1	B	0.57	0.012,80	5.50
2	C	1.18	0.016,48	4.08
1	C	1.24	0.008,55	2.13
3	D	1.32	0.017,95	4.17
1	D	1.44	0.011,68	2.63
2	E	1.89	0.031,33	6.48
3	F	2.22	0.013,40	2.79

TABLE 5

Results of analysis of data for four tasters

		(1) Test 1		(2) Test 2		(3) Test 3	
		Transformed	Untransformed	Transformed	Untransformed	Transformed	Untransformed
		***	***	***	***	***	***
F values	Between juices	281.57	170.13	379.37	244.90	190.58	286.63
	Between sessions	1.86	1.70	1.26	1.26	2.02	2.23
Mean score	Juice A	0.00	0.01	0.00	0.02	.....	.....
	B	0.34	0.36	.....	.....	0.20	0.25
	C	1.42	1.43	1.10	1.10	.....	.....
	D	1.41	1.42	.....	.....	1.25	1.26
	E	.....	.....	1.96	1.96	.....	.....
	F	.....	.....	.....	.....	2.38	2.38

Precision of scoring. The experiment provided evidence on two further points of interest. The first is that the precision of scoring may be greater for some tasters than others; the magnitude of such variation is commonly one of the criteria of panel selection.

It was appropriate to rank tasters' errors of scoring for juices separately. The rankings for juices were tested for consistency by calculating Kendall's coefficient of concordance *(10)*. The details are shown in Table 6.

Variation in level of scoring. Secondly, scoring level changes from session to session. The nature of the frequency distributions shows that it is reasonable to analyse the untransformed scores if the data for juices A and B are omitted. The resulting analyses of variance are shown in Table 7. They are modified split plot analyses with a separate

line in the sub-analysis to accommodate a component of variance for interaction of tasters and sessions. The significance of this effect was substantiated in each test.

TABLE 6

Concordance of errors of scoring of juices for 31 tasters

	(1) Test 1	(2) Test 2	(3) Test 3
Sum of squares, S	17,725.0	10,307.5	11,227.0
Concordance coefficient, $W\left(\frac{12S}{m^2(n^3-n)}\right)$	0.447	0.462	0.503
m	4	3	3
n	31	31	31
$z\left(\frac{1}{2}\log_e\frac{(m-1)W}{1-W}\right)$	0.442	0.270	0.352
v_1	29.5	29.3	29.3
v_2	88.5	58.7	58.7
Tabled value of z (approx.) P = 0.05	.....	0.25	.....
Tabled value of z (approx.) P = 0.01	.....	.....	0.358
Tabled value of z (approx.) P = 0.001	0.432	.....	.....

TABLE 7

Analyses of variance of scores approximately normally distributed

Test		1		2		3	
Juices		C and D		C and E		D and F	
Source of variation	Degrees of freedom	Mean square	F	Mean square	F	Mean square	F
Juices J	1	7.360	9.97 ***	94.388	.....	149.492	458.85 ***
Sessions s	11	0.327	.....	0.916	.....	0.646	.....
Error (a)	11	0.300	.....	0.567	.....	0.326	.....
Total (a)	23						
Tasters t	30	2.994	..... ***	1.979	.....	2.326	5.04 ***
t × J	30	0.738	2.36 **	0.449	1.42 *	0.464	1.39 **
t × s	330	0.464	1.48	0.427	1.35	0.461	1.38
Error (b)	330	0.313	.....	0.317	.....	0.334	.....
Total (b)	743						

One, two, and three asterisks denote significance at P = 0.05, 0.01 and 0.001 probability levels, respectively.

DISCUSSION AND SUMMARY

The differences in bitterness between the juices were very marked; the separate tests were in reasonable agreement except that D scored somewhat higher in test 1 than in test 3. This may be accounted for by the fact that D was the most bitter juice in test 1, so that it seemed slightly more bitter than it was; this is common in taste tests.

It would appear from the correlations between means and variances in Table 4, that the analysis of transformed panel means would be a little more sensitive than that for untransformed means. Comparison of the analyses for the data for 31 tasters shows, however, that the two methods give remarkably consistent agreement. The transformation appears to be

slightly more efficient in test 2, and the reason for this is the extent of the range in the mean scores and the fact that the smallest one is close to the lower limit of the scale. This indicates that only in special circumstances would transformation be expected to give any advantage at all, and this would only be slight. Examination of the data for eight tasters and that for four, summarized in Table 5, indicates that the foregoing conclusions may be taken to apply to the data obtained from an analytical panel of the usual size and say, not less than four to six.

The data of the experiment gave useful information about the ability of the tasters in estimating bitterness. These results provided a good basis for further training and the selection of an analytical panel large enough for other tests. The significant concordance coefficients in Table 6 show that tasters differ widely in their consistency of scoring. The analyses of Table 7 support the hypothesis that tasters do not maintain their individual levels of scoring. It can be shown that this effect does not invalidate juice comparisons. Further details about characteristics of the tasters are given in Appendix I.

The examination of scores by analysis of panel means does not provide a check on the individual performance of panel members. This emphasizes that careful training and panel selection is essential for efficient experimentation.

Acknowledgments

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LITERATURE CITED

1. Bartlett, M. S. The use of transformations. *Biometrics*, **3**, 39 (1947).
2. Boggs, M. M. Sensory methods for measuring differences in food quality: review of literature and proceedings of conference. (Edited by E. H. Dawson and B. L. Harris.) U. S. Department of Agriculture *Agric. Inform. Bull. No. 34*, 94 (1951).
3. Boggs, M. M., and Ward, A. C. Scoring techniques for sulphited foods. *Food Technol.* **4**, 282 (1950).
4. Cartwright, L. C., and Nanz, R. A. The comparative evaluation of spices. *Food Technol.* **2**, 330 (1948).
5. Cochran, W. G. The analysis of variance when experimental errors follow the Poisson or binomial laws. *Ann. Math. Statist.* **11**, 335 (1940).
6. Fisher, R. A. *Statistical Methods for Research Workers.* 10th ed., 1946, Oliver and Boyd, Edinburgh.
7. Fisher, R. A., and Yates, F. *Statistical Tables for Biological Agricultural and Medical Research.* 3rd ed., 1949, Oliver and Boyd, London.
8. Hopkins, J. W. A procedure for quantifying subjective appraisals of odor, flavor, and texture of foodstuffs. *Biometrics.* **6**, 1 (1950).
9. Kendall, M. G. *The Advanced Theory of Statistics.* 2nd ed., 1945, Vol. 1. Charles Griffin & Co. Ltd., London.
10. Kendall, M. G. *The Advanced Theory of Statistics.* 1946, Vol. 2. Charles Griffin & Co. Ltd., London.
11. Salmon, T. N., and Blakeslee, A. F. Genetics of sensory thresholds: variations within single individuals in taste sensitivity for phenyl thiocarbamide. *Proc. Nat. Acad. Sci. U. S.*, **21**, 78 (1935).
12. Williams, E. J. Experimental designs balanced for the estimation of residual effects of treatments. *Aust. J. Sci. Res.* Ser. A **2**, 149 (1949).

APPENDIX I

Grading Tasters in Efficiency of Bitterness Scoring

Individuals are chosen for taste panels by certain criteria. There is no standard practice but it might be said that ability to discriminate between different degrees of bitterness, skill at interpreting the scale, consistency of scoring, and validity of scores are very important. The tasters who participated in the experiment were graded by these criteria.

Overall mean scores for the six juices were accepted as the most reliable information about them. Using numbers of scores as weights, the correlation coefficient of individual means with overall means was calculated for each taster as an index of agreement, r, with the panel and linearity of scoring. These figures are shown in Table 8.

If individual and overall juice means are the same, the linear regression coefficient for dependence of the individual on the panel would be unity. The slopes, b, of such lines are shown in Table 8. When a taster has a line whose slope is greater than unity, this is interpreted to mean that the taster has shown greater than average discrimination. In a similar way, slopes less than unity mean less than average discrimination.

Each of the ten sets of standard deviations of scores in groups of 12 were ranked for tasters and the sums of the ranks for individual tasters calculated. The ranking of these sums is described by Kendall *(9)* as the "best" ranking in the least squares sense. This ranking (sixth column of Table 8) was taken to rank the tasters in order of precision of scoring, the smallest error having rank 1.

TABLE 8
Grading of tasters

Taster	r	Rank of r	b	Rank of b	Error rank	Sum of ranks	"Best" ranking
M.C.	0.9883	7	1.3873	1	11	19	1
K.H.	0.9880	8	1.0952	13	1	22	2
K.S.	0.9866	11	1.1272	9	3	23	3
B.J.	0.9950	5	1.3134	4	16	25	4½
B.C.	0.9812	17	1.2994	6	2	25	4½
S.C.	1.0000	1	1.0146	18	6½	25½	6
J.L.	0.9973	3	0.9997	19	8	30	7
J.C.	0.9770	22	1.3509	2	6½	30½	8
W.E.	0.9939	6	1.1675	8	17	31	9
E.H.	0.9744	24	1.3467	3	9	36	10
J.K.	0.9872	9½	1.3039	5	22	36½	11
F.S.	0.9858	14	0.9685	20	4	38	12
H.M.	0.9872	9½	1.1127	11	21	41½	13
J.H.	0.9776	21	1.1005	10	12	43	14½
J.H.C.	0.9828	16	1.2107	7	20	43	14½
H.E.	0.9999	2	1.0237	15	27	44	16½
J.M.G.	0.9970	4	1.0161	17	23	44	16½
E.A.	0.9793	18	1.0196	16	13	47	18
E.D.	0.9860	12	1.0431	14	26	52	19
P.M.	0.9859	13	0.9076	21	19	53	20
C.R.	0.9782	20	0.8237	24	15	59	21½
T.R.	0.9311	28	0.7583	26	5	59	21½
J.W.	0.9831	15	0.8979	22	24	61	23
D.B.	0.9686	26	0.6631	28	14	68	24
R.O.	0.9335	27	1.0990	12	30½	69½	25
A.A.	0.9789	19	0.8907	23	28½	70½	26
J.M.	0.8890	30	0.4636	31	10	71	27
M.W.	0.9761	23	0.8226	25	25	73	28
W.S.	0.7981	31	0.5214	30	18	79	29
M.B.	0.9731	25	0.7038	27	30½	82½	30
A.H.	0.9265	29	0.6488	29	28½	86½	31

The values of r and b were ranked from highest to lowest. These three rankings had a concordance coefficient, W *(10)*, significant at $P = 0.01$. Hence there was a significant degree of correlation between the abilities of the tasters judged by these criteria. The three ranks were added and ranked to give the overall grading of tasters shown as "best" in Table 8.

One convenient way to check tasters' performance is by graphs in the nature of quality control charts. The chart for variability, if compiled from ranges, will be at the disability conferred on it by the discreteness of the scale, and the non-normality of the scores. Here this difficulty was overcome by ranking standard deviations as mentioned above.

The distribution of the mean score for an individual taster and juice might be expected to approach normality for all juices. The graphs of these means are shown in Figure 2. Validity of score means that a taster must not deviate too far from the

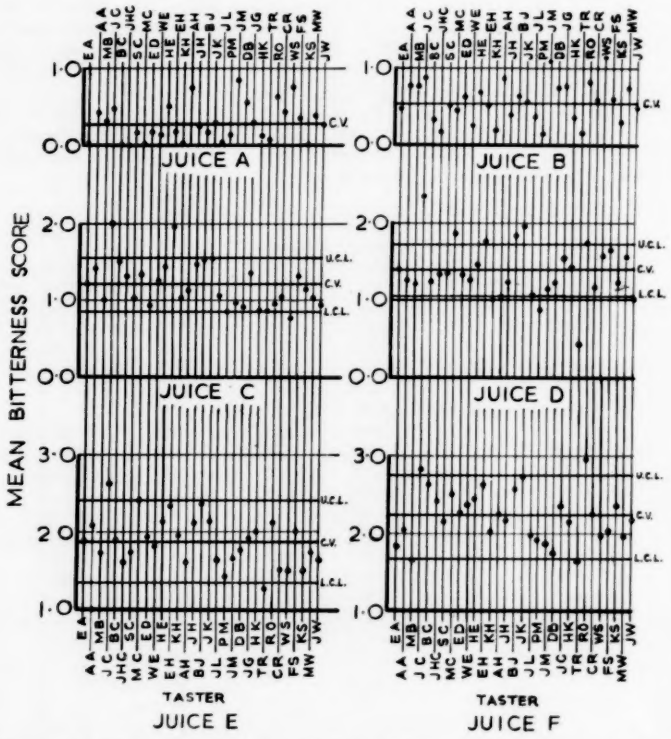

Figure 2. Control chart for mean scores for tasters: central value and upper and lower control limits shown for juices C to F.

"correct" score which must be assumed satisfactorily estimated by the panel mean. For juices C to F the parent distributions seemed near enough to normality to make the usual derivation of upper and lower control limits valid. These limits calculated from total variance for the 1% probability level are shown in Figure 2. Because distributions of scores for juices A and B are too skew to provide valid limits in the usual way, inspection of the diagrams was taken as a sufficient guide. Study of Figure 2 shows how to correct the scoring of tasters and modify the grading in Table 8.

CATHODE RAY IRRADIATION OF CHICKEN MEAT FOR THE EXTENSION OF SHELF LIFE[a, b, c]

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Poultry is sold to the consumer under several different conditions, (a) as freshly eviscerated, (b) as eviscerated, cut up, and refrigerated, (c) as eviscerated, cut up, and frozen, and (d) as a canned heat-processed product.

So far as the eviscerated, cut up, refrigerated product is concerned, the onset of bacterial spoilage limits the storage life to periods of one or two weeks. Some method of processing that extends the storage life of cut-up poultry at refrigerator temperatures above freezing without causing marked changes in the organoleptic properties of the product would be helpful. For economic reasons, a process that would destroy all spoilage bacteria in poultry without markedly affecting organoleptic properties would be even more desirable.

An investigation was initiated, therefore, to determine whether cut-up chicken meat could be treated with high-voltage cathode rays to extend the storage life at refrigerator temperatures above freezing and prevent the growth of spoilage bacteria that might occur at temperatures encountered during shelf storage of canned heat-processed foods.

EXPERIMENTAL PROCEDURES

Preparation of chicken for experiments. The chicken meat used in this investigation came from prepackaged, cleaned, and dressed chickens obtained from a local supermarket. The meat was steam-blanched for 6 to 8 minutes to facilitate separation of the flesh from the bones. The boned meat together with the skin was packed and sealed in quarter-pound sardine cans lined with A-enamel.

To study the effects of different processing treatments prior to irradiation, some of the samples were frozen in the cans. Others were vacuum packed or packed in an inert atmosphere (nitrogen). Still others were precooked or treated with a free-radical acceptor. Some samples received no special treatment.

In the preparation of frozen samples, the chicken meat was sealed within the cans and held overnight at −20°F.

All cans were sealed in a semi-automatic closing machine. For the vacuum-packed samples, the sealing chamber was evacuated to 29 inches of mercury, prior to sealing the can. The pressure within the can as measured by a vacuum gauge was approximately 25 inches of vacuum.

The method of packing under nitrogen gas was as follows: The sealing chamber was evacuated to 29 inches of mercury, after which nitrogen gas under pressure was

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[a] Presented at the Fifteenth Annual Meeting of the Institute of Food Technologists, Columbus, Ohio, June 14, 1955.

[b] Contribution No. 259 from the Department of Food Technology, Massachusetts Institute of Technology.

[c] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 579 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

allowed to enter the evacuated chamber. When the pressure gauge indicated a slightly positive pressure within the chamber, the can was automatically sealed.

In one test on precooked chicken, the meat was broiled in an electric oven and then canned. In another test, the meat was steamed at atmospheric pressure and canned.

The treatment for studies with free-radical acceptors was as follows: An aqueous solution of free-radical acceptors was pumped with a hypodermic needle into the intact muscles of a chicken to provide a concentration by weight of 0.5% of monosodium glutamate and 0.3% of sodium fumarate, or 1% sodium ascorbate. The bird was then held at 36–40°F. for 18 to 24 hours to permit diffusion of the chemical solution into the tissues. The amount of the solution was such that the weight of the pumped bird was not increased by more than 5%.

Irradiation technique. A Van de Graaff accelerator *(5)* rated at 3 million electron volts (m.e.v.) was the source of high-voltage cathode rays. Samples to be irradiated were placed on a motor-driven belt, which was traversed by the electron beam from the accelerator. For chicken packed in sardine cans, irradiation from both sides through the shortest dimension of the can was required. It was calculated that with the use of the 3 m.e.v. electron beam and with irradiation of the food product on both sides, the thickness of the food product inside the can should be 16.9 mm. if the most efficient utilization of the beam were to be obtained.

The dose rate was checked by using the ferrous-ferric system of dosimetry *(7)*.

A run was considered satisfactory if the requested dose and the calculated dose did not differ by more than 10%.

Organoleptic tests immediately after irradiation. To study the effects on flavor of cathode ray irradiation in the dose range expected to give the desired degree of bacterial destruction, organoleptic tests were made on the variously treated and irradiated samples immediately following irradiation (less than 24 hours). During the short interval between irradiation and organoleptic tests, the irradiated foods were kept at 36–40°F. Samples were prepared for tasting by broiling them in an electric oven in all instances except one, when the samples were steam cooked. No seasoning was added during or after cooking, previous to panel tests.

A taste panel composed of 20 members was used to evaluate the cooked meat. The evaluation was done by the hedonic scale method. A score sheet was used with a scale ranging from 1 to 9, to indicate the degree of acceptability as follows:

9 — like extremely
8 — like very much
7 — like moderately
6 — like slightly
5 — neither like nor dislike
4 — dislike slightly
3 — dislike moderately
2 — dislike very much
1 — dislike extremely

According to this scale, a score of 5 or above indicates acceptability. Mean scores were determined by summing the individual ratings for a given sample and dividing by the number of judges. Calculation of the significance of the difference between the means was done by the "t" method *(3)*.

Bacteriological methods. Standard plate counts for aerobic bacteria were made in accordance with standard methods of analysis *(1)*. The initial dilution was prepared by disintegrating 22 g. of the meat with 198 ml. of chilled, sterile, distilled water in a sterile Osterizer for 5 minutes. Appropriate decimal dilutions were made and plated in duplicate on tryptone glucose extract agar. Plate cultures were incubated at 30°C. for 48 to 72 hours before colonies were counted. Since the lowest dilution plated was 1:10, if the number of colonies appearing on these plates or the plates of higher dilution was fewer than 30, the count was reported as less than 300 per gram.

The Most Probable Numbers (MPN) of anaerobic organisms were determined by inoculating series of 5 tubes containing sterile liver broth media with 1-ml. portions of an appropriate dilution of the sample and stratifying these cultures with sterile melted vaseline. This procedure was then repeated for two successively greater dilutions. Following an incubation period of 2 weeks at 37°C., the tubes showing gas

production and growth, or growth alone, were noted and counts of putrefactive and total anaerobes were calculated from MPN Tables *(1)*.

Determination of sterilizing dose. To determine the dose of cathode rays required for sterilization, the natural bacterial flora were used as the source of contamination. The meat was packed and sealed in sardine cans and irradiated at levels of 1×10^6, 1.5×10^6, 2×10^6, or 2.5×10^6 rep. Bacterial counts for both aerobes and anaerobes were made on all samples immediately after irradiation and following incubation at 37°C. for 1 week and 2 weeks. Non-irradiated samples had developed into hard swells after 1 week's incubation, and were not held any longer.

To determine the sterilizing dose when *Clostridium sporogenes* was used as the contaminating source, chicken meat was first sterilized by being treated with a dose of 3×10^6 rep. Then the meat was aspetically cut into small pieces and disintegrated in a sterile Waring blender. A spore suspension of *Cl. sporogenes* was next added to the puréed chicken, and the two were thoroughly blended. Approximately 1.5-g. portions were pipetted into individual containers, which measured 30 mm. in length, 12 mm. in width, and 6 mm. in height and which were made from aluminum foil. Each container was heat-sealed in a cellophane envelope within a cellophane envelope, and the samples were irradiated at dose levels ranging from 1×10^6 rep to 3.5×10^6 rep in increments of 0.5×10^6 rep. Ten samples were irradiated at each dose level.

After irradiation, the contents of each container, including non-irradiated samples, were aseptically transferred to a culture tube (25 mm. × 190 mm.) containing sterile thioglycollate broth, and this was stratified with sterile melted vaseline. After an incubation period of 2 weeks at 37°C., those tubes that showed gas production were regarded as positive evidence that the inoculum contained survivors of the irradiation.

Determination of D_o. To determine the D_o, that is, the dose that results in 37% survival *(3)*, chicken meat was sterilized and contaminated with a spore suspension of *Cl. sporogenes* just as described in the preceding section. Fifteen-gram portions were filled into aluminum cups 60 mm. in diameter and 15 mm. in height, and these in turn were sealed in sardine cans.

Some samples were chilled to a temperature of approximately 50°F. and irradiated at various doses of 250,000 rep, 500,000 rep, and 750,000 rep. Other samples were irradiated in the frozen state, with the same doses. Nine samples were cultured at each dose level, including non-irradiated controls.

The number of survivors of each irradiation dose was determined by a direct count. This was done by placing 1 ml. of an appropriate dilution of the sample in a pyrex tube 10 mm. in diameter and 200 mm. in length, adding 0.5 ml. of $NaHCO_3$, 10 ml. of Andersen's media *(2)*, and stratifying with sterile vaseline. Tubes were incubated at 37°C. for 3 days before colonies were counted.

Individual D_o values were calculated for each dose level by use of the equation:

$$n = n_o e^{-D/D_o}$$

where n is the number of organisms surviving the applied dose D, n_o represents the initial number of organisms per gram, and D_o is the dose representing 37% survival. The average D_o was obtained for the various irradiation levels and also the confidence limits at the 95% level of significance.

Storage tests. Chicken meat was packed in sardine cans and irradiated at two different levels and under different conditions.

One set of samples was irradiated at room temperature with either 0.8×10^6 rep or 2×10^6 rep. Samples irradiated with 0.8×10^6 rep were stored at 36–40°F. and samples irradiated with 2×10^6 rep at 68°F. The length of storage was 6 weeks. Non-irradiated controls were stored along with irradiated samples just for comparison of storage life. However, non-irradiated controls for tasting were kept at 0°F. Bacterial, chemical, and organoleptic tests were made immediately following the irradiation and after storage for 3 and 6 weeks. Samples that had been irradiated with 2×10^6 rep were incubated at 37°C. for bacterial analysis.

Another set of samples was irradiated in the frozen state with either 0.8×10^6 rep or 2×10^6 rep and then handled in the same manner for the storage studies as was described for the first set, except that the storage period was of 4 weeks' duration.

Chemical tests. To determine some chemical changes induced by irradiation, especially when irradiation followed various processing conditions, chicken meat was either

air-packed, vacuum-packed, or frozen in sardine cans and irradiated with 2×10^6 rep. Immediately after irradiation, measurements were made on irradiated and non-irradiated samples for hydrogen ion concentration, peroxide number, and free fatty acid content.

Chemical studies on irradiated samples undergoing storage included determinations of the peroxide number, the thiobarbituric acid value *(6)*, and the free fatty acid content.

RESULTS AND DISCUSSION

When chicken meat was irradiated in an air atmosphere with a dose of 2.5×10^6 rep, its flavor was significantly different from the flavor of non-irradiated samples (Table 1). Irradiation with a dose of 2×10^6 rep gave variable results, but in the majority of the trials there was no significant difference, flavorwise, between samples irradiated at 2×10^6 rep and controls.

It was found possible to irradiate chicken meat with a dose of 2.5×10^6 rep without causing a significant flavor change, provided the meat prior to irradiation was either frozen, vacuum packed, nitrogen packed, or treated with a solution of the free-radical acceptors, sodium glutamate together with sodium fumarate.

The off-flavor development due to irradiation at a level of 2.5×10^6 rep was not obviated by steam-cooking or oven-broiling the meat prior to irradiation.

The taste panel had no significant preference for either white meat of chicken or the dark meat after both had been irradiated with a dose of 2×10^6 rep.

Results of bacterial tests made on triplicate samples of chicken meat that had been irradiated with doses ranging from 1×10^6 rep to 2.5×10^6 rep indicated that commercial sterility was not attained in samples irradiated with 1.5×10^6 rep or less but was attained in those samples treated with 2×10^6 rep or more. Samples irradiated with 1×10^6 rep or 1.5×10^6 rep developed into hard swells after 1 week of incubation at 37°C. This was also true of non-irradiated controls, whereas an insignificant bacterial count prevailed among samples irradiated with 2×10^6 rep or 2.5×10^6 rep after 2 weeks' incubation at 37°C. These data are presented in Table 2.

The average D_0 values for *Cl. sporogenes* in chicken irradiated in air at 50°F. or in the frozen state were significantly different when the dose levels were 0.25×10^6 rep, 0.5×10^6 rep, and 0.75×10^6 rep (Table 3). These results indicate that during treatment with 0.25×10^6 rep, 0.5×10^6 rep or 0.75×10^6 rep and most probably at higher doses, spores of *Cl. sporogenes* are more radio-resistant in the frozen state than in the non-frozen state.

With samples of comminuted chicken meat that contained an initial inoculum of 260 spores of *Cl. sporogenes* per gram and that were irradiated in 1.5-gram portions in small aluminum "boats" at various dose levels and then transferred to tubes containing sterile thioglycollate broth, the results obtained after 2 weeks' incubation at 37°C. were as follows:

Of 10 samples that were not irradiated, all showed positive growth (gas production). Of 10 samples irradiated with 1×10^6 rep, four showed growth. Of 10 samples irradiated with either 1.5×10^6 rep, 2×10^6 rep, 2.5×10^6 rep, 3×10^6 rep, or 3.5×10^6 rep, no growth was evidenced in any sample.

TABLE 1

Flavor evaluation of non-irradiated and irradiated chicken treated in various manners

Treatment	Irrad. dose (rep)	Mean flavor score	Significance at 5% level
None	None	6.16	.....
None	2×10^6	6.00	Not significant
Frozen	2×10^6	5.79	Not significant
None	None	6.55	.....
None	2.5×10^6	5.04	Significant
Frozen	2.5×10^6	6.44	Not significant
None	None	6.94	.....
Frozen	None	7.21	Not significant
Frozen	2.5×10^6	6.05	Not significant
None	None	5.77	.....
None	2×10^6	6.27	Not significant
Pre-broiled	2×10^6	4.66	Not significant
None	None	7.26	.....
Pre-broiled	None	7.11	Not significant
Pre-broiled	2.5×10^6	4.00	Significant
None	None	6.70	.....
None	2.5×10^6	3.95	Significant
Pre-steamed	2.5×10^6	3.65	Significant
None	None	6.68	.....
None	2×10^6	5.00	Significant
Vacuum packed	2×10^6	5.63	Not significant
None	None	7.33	.....
None	2×10^6	4.72	Significant
Vacuum packed	2×10^6	6.00	Significant
None	None	6.84	.....
None	2.5×10^6	5.10	Significant
Vacuum packed	2.5×10^6	6.00	Not significant
None	None	6.83	.....
None	2×10^6	6.72	Not significant
Nitrogen packed	2×10^6	5.77	Not significant
None	None	6.30	.....
Nitrogen packed	None	6.35	Not significant
None	2.5×10^6	4.60	Significant
Nitrogen packed	2.5×10^6	5.25	Not significant
None	None	6.45	.....
None	2×10^6	5.45	Not significant
Injected with solution of sodium fumarate and monosodium glutamate	2×10^6	6.40	Not significant
None	None	6.25	.....
None	2.5×10^6	5.05	Significant
Injected with solution of sodium fumarate and monosodium glutamate	2.5×10^6	5.80	Not significant
None	None	6.60	.....
None	2.5×10^6	4.95	Significant
Injected with solution of sodium ascorbate	2.5×10^6	6.20	Not significant
None (white meat)	2×10^6	6.25	.....
None (dark meat)	2×10^6	6.45	Not significant

TABLE 2

Bacterial content of canned chicken meat irradiated with various doses and stored at 37°C.

Irrad. dose (rep)	Bacterial count[1]	Number of organisms per gram		
		Initial	1 week	2 weeks
Control	SPC	1,800,000	34,000,000	.....
	TA	18,000	18,000,000	.....
	PA	11,000	6,500,000	.....
1×10^6	SPC	<300	67,000,000	.....
	TA	120	1,800,000	.....
	PA	1	1,800,000	.....
1.5×10^6	SPC	<300	35,000,000	.....
	TA	18	1,800,000	.....
	PA	18	1,800,000	.....
2×10^6	SPC	<300	<300	<300
	TA	31	205	28
	PA	3	2	1
2.5×10^6	SPC	<300	<300	<300
	TA	12	27	643
	PA	0	1	2

[1] SPC = Standard plate count.
TA = Total anaerobes.
PA = Putrefactive anaerobes.

TABLE 3

Calculated D_0 values for Cl. sporogenes suspended in chicken meat irradiated in air at 50°F., or in the frozen state

Number of samples	Irrad. condition	Irrad. dose (rep)	Calculated mean D_0 value (rep)	Confidence limits at 95% level	Significance
8	Air (50°F.)	250,000	604,000	±319,000	.....
9	Frozen	250,000	1,083,000	±660,000	Significant
8	Air (50°F.)	500,000	226,000	±28,600	.....
9	Frozen	500,000	310,000	±29,000	Significant
8	Air (50°F.)	750,000	166,000	±14,900	.....
9	Frozen	750,000	224,000	±19,800	Significant

TABLE 4

Irradiation effects under various processing conditions on pH, peroxide number, and free fatty acid content of chicken

Treatment	Irrad. dose (rep)	pH	Peroxide No. (mM/Kg)	Free fatty acid as oleic acid
				%
None	None	6.60	2.5	0.73
None	2×10^6	6.61	2.6	0.78
Vacuum packed	2×10^6	6.61	3.6	0.80
Frozen	2×10^6	6.59	2.7	0.74

TABLE 5

Results of chemical studies on chicken before and after irradiation and storage

Storage		Irradiation condition	Irrad. dose (rep)	Peroxide number (mM/Kg)	TBA value[1]	Free fatty acids (%)
Weeks	Temp. (°F.)					
0	.....	Irradiated in frozen state	None	1.4	0.494	0.50
			0.8×10^6	1.5	0.499	0.49
			2×10^6	1.5	0.706	0.51
0	.....	Irradiated at room temp.	None	1.5	0.301	0.56
			0.8×10^6	1.6	0.426	0.53
			2×10^6	1.6	0.440	0.54
6	36–40	Irradiated at room temp.	0.8×10^6	2.7	0.925	0.60
	68		2×10^6	3.2	0.991	0.66

[1] TBA = Thiobarbituric acid value, expressed as optical density.

Chicken that was either air-packed, vacuum-packed, or frozen did not undergo any significant change in pH, peroxide number, or free fatty acid content after having received a dose of 2×10^6 rep (Table 4).

A noticeable change in the thiobarbituric acid value occurred in samples that were irradiated at room temperature or in the frozen state with a dose of 2×10^6 rep.

With samples that had been irradiated (room temperature) with a dose of 0.8×10^6 rep and stored at 36–40°F., or samples that had been irradiated (room temperature) with a dose of 2×10^6 rep and held at 68°F., there were increases in peroxide number, free fatty acid content, and thiobarbituric acid value after 6 weeks of storage, but there was no indication of rancidity in any of the samples (Table 5).

TABLE 6

Bacterial content and organoleptic evaluation of chicken irradiated at room temperature and stored at various temperatures

Storage		Irrad. dose (rep)	Bacteria/g.[1]			Mean flavor score	Significance at 5% level
Weeks	Temp. (°F.)		SPC	TA	PA		
0	Room temp.	None	11,700	7,700	485	6.25	Significant
		0.8×10^6	<300	45	3	4.90	
0	Room temp.	None				6.20	
		2×10^6	<300	21	1	5.85	Not sig.
3	0	None				6.55	
	36–40	0.8×10^6	<300	47	8	5.45	Significant
3	0	None				6.85	
	68	2×10^6	<300[2]	44[2]	7[2]	5.55	Significant
6	0	None				6.50	
	36–40	0.8×10^6	<300	44	6	5.30	Significant
6	0	None				6.30	
	68	2×10^6	<300[2]	6[2]	4[2]	4.80	Significant

[1] SPC = Standard plate count, TA = Total anaerobes, PA = Putrefactive anaerobes.
[2] Incubated at 37°C.

In relation to organoleptic studies made on stored irradiated chicken, samples irradiated at room temperature with a dose of 0.8×10^6 rep and stored at 36–40°F., or irradiated with a dose of 2×10^6 rep and stored at 68°F., received significantly lower flavor scores than frozen controls at the end of 3 and 6 weeks of storage (Table 6).

When the chicken was irradiated in the frozen state, samples that had been irradiated with a dose of 0.8×10^6 rep were not significantly different in flavor from non-irradiated samples immediately after irradiation and following a 2-week storage period at 36–40°F., but after 4 weeks of storage there was a significant flavor difference (Table 7).

TABLE 7

Bacterial content and organoleptic evaluation of chicken frozen, irradiated, and stored at various temperatures

Storage		Irrad. dose (rep)	Bacteria per gram			Mean flavor score	Significance at 5% level
Weeks	Temp. (°F.)		Standard plate count	Total anaerobes	Putrefactive anaerobes		
0	Room temp.	None	422,000	18,000	9,280	6.95	
		0.8×10^6	2,600	282	0	6.45	Not sig.
0	Room temp.	None	.....	.....	.....	6.30	
		2×10^6	<300	43	0	6.10	Not sig.
1	36–40	None	48,000,000	1,800,000	1,800,000	.....	.....
2	0	None	.....	.....	.....	6.10	
	36–40	0.8×10^6	1,000	1,800	0	6.00	Not sig.
2	0	None	.....	.....	.....	6.25	
	68	2×10^6	<300[1]	604[1]	3[1]	5.55	Not sig.
4	0	None	.....	.....	.....	6.60	
	36–40	0.8×10^6	1,500	640	0	5.50	Significant
4	0	None	.....	.....	.....	7.05	
	68	2×10^6	<300[1]	39[1]	1[1]	5.80	Significant

[1] Incubated at 37°C.

Samples irradiated in the frozen state with a dose of 2×10^6 rep were not significantly different in flavor from non-irradiated samples immediately after irradiation and following a 2-week storage period at 68°F., but they were significantly different after 4 weeks of storage. However, even though the irradiated samples were significantly different in flavor after 4 weeks of storage, they were still considered to be acceptable in flavor since the hedonic rating was above 5.0.

The storage life of the non-irradiated control samples of chicken, which were prepared and packaged in the same manner as the irradiated samples, was less than 1 week when the samples were held at 36–40°F. (Table 7) and also less than 1 week when held at 68°F. (Table 8).

CONCLUSIONS

In four out of six separate trials, samples of chicken meat irradiated with high-voltage cathode rays at levels of 2×10^6 rep without pretreatment were not significantly different in flavor from controls. However, in

TABLE 8

Bacterial content of irradiated and non-irradiated chicken held at 68°F.

Storage period (weeks)	Dose (rep)	Standard plate count	Total anaerobes	Putrefactive anaerobes
0	None	1.8×10^8	18×10^3	11×10^3
	2×10^6	<300	188	9
1	None	88×10^6	18×10^6	18×10^6
	2×10^6	<300	619	13
2	2×10^6	<300	715	3

six separate trials with a dose of 2.5×10^6 rep, the irradiated samples were significantly different from the controls in every case.

In three tests for each type of pretreatment, vacuum-packed, frozen, or treated with free-radical acceptors, samples of chicken meat irradiated at levels of 2×10^6 rep or 2.5×10^6 rep were not significantly different from controls.

In tests in which samples of chicken containing a natural microbial flora were packed in hermetically sealed cans, irradiated, and stored at 68°F. or higher for a period of two weeks, commercial sterility was attained by application of 2.0×10^6 rep. Commercial sterility was considered to have been attained when fewer than 300 aerobic bacteria and fewer than 100 putrefactive anaerobic bacteria were present in the product after incubation. Since sterility with irradiation treatment of this kind is a function of the number and kinds of bacteria present, conditions might arise in which sterility would not be attained with a dose of 2.0×10^6 rep.

In chicken containing a concentration of 260 spores of *Cl. sporogenes* per gram, sterility was achieved with a dose of 1.5×10^6 rep. Ten inoculated 1.5-gram samples were used for this test.

No significant changes occurred in the hydrogen ion concentration, the peroxide number, or the free fatty acid content of chicken meat irradiated with 2×10^6 rep.

Samples of chicken meat irradiated in the frozen state with 0.8×10^6 or 2×10^6 rep and stored at 36–40°F. were not significantly different in flavor from frozen controls after two weeks of storage but were significantly different after four weeks. However, the irradiated samples were still considered acceptable in flavor after the 4-week storage period at these temperatures.

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LITERATURE CITED

1. American Public Health Association. *Standard Methods for the Examination of Dairy Products.* 9th ed., 1948, New York, N. Y.
2. ANDERSEN, A. A. A rapid plate method of counting spores of *Cl. botulinum*. *J. Bact.*, 62, 425 (1951).
3. LEA, D. E. *Actions of Radiations on Living Cells.* 1947, Macmillan Co., New York, N. Y.
4. PATERSON, D. D. *Statistical Technique in Agricultural Research.* 1st ed., 1939, McGraw-Hill, New York, N. Y.
5. TRUMP, J. G., AND VAN DE GRAAFF, R. J. Irradiation of biological materials by high energy roentgen rays and cathode rays. *J. Appl. Phys.*, 19, 599 (1948).
6. TURNER, E. W., PAYNTER, W. D., MONTIE, E. J., BESSERT, M. W., STRUCK, G. M., and OLSON, F. C. Use of the 2-thiobarbituric acid reagent to measure rancidity in frozen pork. *Food Technol.*, 8, 326 (1954).
7. WEISS, J. Chemical dosimetry using ferrous and ceric sulfates. *Nucleonics*, 10 (7), 28 (1952).

THE DIRECT MICROSCOPIC ENUMERATION OF BACTERIA IN THE WASHINGS OF GREEN BEANS[a]

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The microscopic method for the enumeration of bacteria on frozen vegetables as presented by the Committee on Microbiological Examination of Foods *(3)* was designed as a procedure to detect undesirable unsanitary conditions in the processing plant, or improper practices in handling the raw product and the frozen product in storage and in transit. Although low bacterial counts do not assure the absence of harmful bacteria in the foods undergoing examination, the probability of their presence is reduced materially; low bacterial content also reflects favorably from the standpoint of esthetic considerations.

When applied to vegetables such as peas, and to shellie and lima beans, the method as presented is of value, for these products are sterile until threshed, and microorganisms present at the time of microscopic examination are indicative of the degree of attention given to the factors of storage and of sanitation. The microorganisms are limited in variety of flora, are easily removed by the washing procedure preparatory to making the smear, and are readily stained.

In contrast, the normal surface of the green bean as it occurs in nature becomes part of the edible, finished portion. As reported initially by Burri *(2)*, Duegelli *(4)*, and others (*cf.* the review by Thaysen and Galloway [*12*]), such surfaces contain epiphytic bacteria in numbers which may range into many millions per unit area. Together with the transient microorganisms, the morphology of the flora is diverse. As Starkey *(10)* has reported for the bacteria of the rhizosphere, so also many of the plant epiphytic flora are small and coccoid, and do not stain well with many stains commonly used in bacteriology.

It is the objective of an adopted procedure to reveal as many microorganisms as possible. Thus, the selection of the dye or the stain and the procedure to be followed may not be a matter of choice left in the hands of the investigator, as is indicated by Tanner *(11)*; rather, it should be that stain and that procedure which best accomplishes the objective. In another area, specifically that of staining smears of milk, it has been shown by Levine and Black *(6)*, Olson and Black *(8)*, and Brandstein and Mundt *(1)* that not all staining procedures yield comparable results.

Much of the present work involved a study of stains and procedures adaptable to the staining of bacteria in suspensions prepared from green beans. The stain then was evaluated as a means of quality control.

MATERIALS AND METHODS

The size of the sample, the quantity of suspending fluid, and the standardization of the microscope were those established by the Committee on Microbiological Examination

[a] Published with the approval of the Director of the Experiment Station.

of Foods *(3)*. During the course of the work it was observed that more accurate results were obtained if the prescribed area were delimited with etched lines, if 0.1 ml. of 1% solution of a surface tension depressant [b] were added to the suspending fluid, and if the droplet were spread with the tip of the Breed pipette, rather than with a bent needle. It also was desirable to swirl the flask vigorously prior to taking each sample droplet, when more than one smear was to be prepared from the same flask.

Since the majority of the bacteria on the slide lose the dye when excessive amounts of water are used in washing, it became standard procedure to wash with not over 2 ml. of water per smear on the slide.

Stains and procedures included the Gram stain and its components, aqueous, alcoholic, or carbolated crystal violet, basic fuchsin, safranin, acid fuchsin, methylene blue, and acidified methylene blue, Barlow's stain, alkaline congo red, and the AWF stain (Levine and Black [7]).

Both irregularly-shaped, and also quite small, intensely stained bodies were seen on some smears. To demonstrate the identity of these as bacteria, numerous colonies from platings on tryptone glucose extract agar were picked in parallel to nutrient broth and to an infusion prepared with a segment of green bean in 10 ml. of water. On examination of Gram stained preparations, it was seen that irregularity of shape and diminution of size were common among bacteria grown in the infusion medium.

Little difference in numbers of bacteria was obtained when the stains recommended by the Committee were compared. Therefore, Gray's stain *(5)* was selected as the reference stain. Of the various stains included in the study, only one proved satisfactory. Only the results obtained with this stain will therefore be presented.

RESULTS

The Hucker modification of the crystal violet stain, applied for 5 minutes, gave the most satisfactory results. Bacteria are more intensely stained than is the non-bacterial debris, definition of the cell is good, and very small bacteria are recognizable. The efficacy of the stain, when used with a surface tension depressant in the suspending fluid, is demonstrated in Table 1. Ten smears were prepared from each of 3 samples,

TABLE 1

Evaluation of crystal violet stain as applied to washings of green beans

	Raw bean square smear	Processed bean square smear	Raw bean circular smear
Mean, number per gram	39,300,000	48,160,000	20,320,000
Maximum count	49,000,000	52,000,000	21,620,000
Minimum count	34,400,000	43,500,000	19,400,000
Number of smears	10	10	10
Standard deviation	3,583,000	2,749,000	767,600
Coeff. of variation	9.1		3.8
Standard error	1,133,000	870,000	243,000
Coeff. of variation	2.9		1.2
F value between coeff. of variation	2.4		

two of unprocessed beans and one of processed, frozen beans. The summarized data present the mean, maximum, and minimum counts of bacteria per gram of sample.

The standard deviation (coefficient of variation, 9.1%) of counts obtained from smears in form of a square, 1 cm.^{2}, is somewhat larger than that of counts obtained from circular smears (coefficient of variation, 3.8%). Similarly, the standard error, with coefficients of variation 2.9% and 1.2%, respectively, is somewhat greater. The coefficients of variation are not significantly different, however, having an F value of 2.4.

The superiority of the crystal violet stain over the double dye stain is demonstrated in Table 2, in which the data obtained with 14 independent samples are summarized.

[b] Santomerse.

TABLE 2

Comparison of crystal violet with the double dye stain applied to 14 different samples of unprocessed and processed green beans

	Crystal violet stain	Double dye stain
Mean, number per gram	4,960,000	1,364,000
Maximum count	10,100,000	2,360,000
Minimum count	1,000,000	580,000
Number samples	14	
Standard deviation	2,531,000	602,000
Standard error	676,000	161,000
Standard error of difference	693,000	
"t" value	5.16	

The probability that the 2 stains would give comparable results is less than 1%.

The probability that comparable results will be obtained with the addition of a surface tension depressant to the suspending fluid is greater than 50%. It provided a slightly clearer field, and somewhat better differentiation between bacteria and non-bacterial debris was noted, and its use was retained. The addition of the surface tension depressant to suspensions to be stained with the double dye stain did not alter the results materially ($t = 0.093$, $d/F = 9$). For the sake of brevity, tables presenting these data are omitted.

The very small bacteria generally are not abundant when stains other than the crystal violet are used. However, the differences in counts are not due entirely to the fact that only this group is brought into prominence. Typical data from one series of counts, presented in Table 3, show that rods of all categories are numerically more abundant, although on a percentage basis the larger cells may occur more frequently with the double dye stain. Less conclusive, although indicative, evidence is also presented by the numbers of large and small cocci detected by the two procedures. Although the differences are consistent, it is recognized that the element of distribution must be considered, particularly when the difference is of the magnitude of one or two cells per 100 fields, and the observed area of the smear is 0.1% of the total area.

Morphologic forms other than those recorded in Table 3 include diamond-shaped and square patterns of tetrads, reminiscent of *Gaffkya* and of *Pediococcus (9)*; curved rods, indicative of *Vibrio;* serpentine forms which appeared to be single cells but which could have been chains of *Vibrio* in close attachment; very occasionally a bacterial spore; and large, rectangular or tapering and budding cells which were probably yeasts. None were sufficiently numerous to tabulate.

Counts were made of various lots of beans processed in the laboratory, or of samples taken from processing lines, before processing and immediately after blanching and cooling. Results of 10 such comparisons are presented in Table 4.

TABLE 4

Per cent retention of epiphytic bacteria per gram of beans on processing

Bacteria per gram		Per cent retention	Remarks
Unprocessed	Blanched and cooled		
5,100,000	3,900,000	74.6	Not irrigated
3,580,000	1,600,000	46.7	Not irrigated
10,100,000	7,600,000	75.3	Not irrigated
7,600,000	4,800,000	63.0	Not irrigated
20,500,000	12,100,000	59.1	Irrigation not known
19,500,000	11,900,000	60.0	Irrigation not known
38,000,000	19,000,000	52.1	Irrigated
36,100,000	16,800,000	46.3	Irrigated
61,500,000	41,800,000	68.0	Irrigated
31,400,000	20,200,000	64.4	Irrigated

TABLE 3

Morphologic distribution of bacteria in 100 fields each of slides prepared from unprocessed and processed green beans stained with crystal violet and double dye stains

Morphology	Unprocessed				Blanched and Cooled				Frozen			
	Crystal violet stain		Double dye stain		Crystal violet stain		Double dye stain		Crystal violet stain		Double dye stain	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Small fine rods	333	61.5	22	33.3	325	62.7	39	36.4	235	62.8	39	39.0
Short blunt rods	148	27.4	25	38.0	167	32.2	57	53.4	114	30.5	52	52.0
Long coarse rods	21	3.9	6	9.2	7	1.4	3	2.8	7	1.9	3	3.0
Long fine rods	32	5.9	10	15.0	14	2.7	6	5.6	12	3.2	4	4.0
Large cocci	2	0.4	1	1.5	2	0.4	1	0.9	2	0.5	1	1.0
Small cocci	5	0.9	2	3.0	3	0.6	1	0.9	4	1.1	1	1.0

The numbers of bacteria per gram of unprocessed beans appear to be directly related to climatic factors, for the counts of bacteria of the beans grown on non-irrigated soil ranged generally from 10,000,000 per gram downward, whereas counts made on beans grown on irrigated land were as high as 61,500,000 per gram.

The numbers of bacteria per gram are reduced as the result of processing, by values ranging between 24.7% and 53.7%, with no decrease predictable to factors observed in this work. The figures of Table 3, for which the slides were prepared from samples taken in processing sequence, indicate that all morphologic groups are reduced in number. No decrease is consistent with any specific group. This would conform to the concept of the degree of adhesion between epiphytic bacteria and the substrate upon which they reside.

DISCUSSION

Apparently, no one staining procedure which will yield maximal numbers of bacteria per unit can be applied to an all-inclusive group of products as vegetables. Some vegetables are sterile until harvest, others contain an epiphytic flora with widely diverse characteristics. This study was limited to the bacteria of green beans, and it would be unwise, with the limited knowledge available, to state that crystal violet stain could or should be applied to vegetables other than the bean. On the other hand, rather than be given a free choice of stains when bacteria on vegetables are to be studied, the investigator should employ the stain which will yield maximal numbers; therefore, the need for an extension of this study is indicated.

The presence of epiphytic flora tends to limit severely, if not eliminate entirely, the use of the direct microscopic procedure for the examination of certain vegetables. Vaughn *et al. (13)* state that several species of Gram negative, rod-shaped bacteria grow freely on the surfaces of equipment in the processing plant. It has been observed locally over the period of years that even though postblanching contamination may be excessive, the numbers of micrococci are minimal, and streptococci, as determined by media containing azide, are seldom as numerous as 10,000 per gram of processed bean.

It would be impossible, therefore, to differentiate between the epiphytic bacteria persisting through processing, and those arising as a result of laxity in sanitation, storage, or growing as the result of delay in freezing. This is more likely today than it was several years ago, for operators of freezing plants give preference to beans grown on irrigated fields, and on these beans the epiphytic population is high.

SUMMARY AND CONCLUSIONS

Hucker's modification of crystal violet stain, applied for five minutes, is superior to other stains investigated, when bacteria in preparations of green beans are to be counted. Bacteria are distinguished readily, and the counts are reproducible.

A varying percentage of the epiphytic flora of the raw product persists during processing. In general, these cannot be distinguished morphologically from those gaining access to the product after blanching. Therefore, the direct microscopic examination when applied to frozen green beans is of little value in determining adequacy of plant sanitation, rapidity of freezing, or proper conditions of storage or of transit.

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LITERATURE CITED

1. BRANSTEIN, M., AND MUNDT, J. O. An analysis of several stains used and proposed for the direct microscopic enumeration of the bacteria in milk. *Food Technol.*, **3**, 324-326 (1949).
2. BURRI, R. Die Bakterienvegetation auf der Oberflache normal entwickelter Pflanzen. *Zentralbl. Bakt. Abt.* II, **10**, 756-763 (1903).
3. COMMITTEE ON MICROBIOLOGICAL EXAMINATION OF FOODS. Microbiological examination of foods. Tentative methods for the microbiological examination of frozen foods. *Am. J. Publ. Health*, **36**, 322-335 (1946).
4. DUEGELLI, MAX. Die Bakterienflora gesunter Samen and daraus gezogener Keimpflanzehen. *Zentralbl. Bakt. Abt.* II, **13**, 56-63 (1904).
5. GRAY, P. H. H. Two-stain method for direct bacteria count. *J. Milk Technol.*, **6**, 76 (1943).
6. LEVINE, B. S., AND BLACK, L. A. A comparative study of commonly used staining procedures for the direct microscopic examination of milk. *J. Milk Food Technol.*, **11**, 139-148 (1948).
7. LEVINE, B. S., AND BLACK, L. A. Newly proposed staining formulas for the direct microscopic examination of milk. *Am. J. Publ. Health*, **38**, 1210-1218 (1948).
8. OLSON, J. C., AND BLACK, L. A. A comparative study of stains proposed for the direct microscopic examination of milk. *J. Milk Food Technol.*, **14**, 49–51 (1951).
9. PEDERSON, C. S., ALBURY, MARGARET, AND BREED, R. S. Pediococcus cerevisiae, the beer sarcina. *Wallerstein Lab. Commun.*, **17**, 7-18 (1954).
10. STARKEY, R. L. Some influences of the development of higher plants upon the microorganisms of the soil. VI. Microscopic examination of the rhizosphere. *Soil Sci.*, **45**, 207-249 (1938).
11. TANNER, F. W. *Laboratory Manual and Work Book in Microbiology of Foods.* 1950, Garrard Press, Champaign, Ill.
12. THAYSEN, A. C., AND GALLOWAY, L. D. *Microbiology of Starch and Sugars.* 1930, Oxford University Press, London.
13. VAUGHN, R. H., LEVINE, M., AND SMITH, H. A. A buffered boric acid lactose medium for enrichment and presumptive identification of *Escherichia coli*. *Food Research*, **16**, 10-19 (1951).

A STUDY OF THE ACID-FORMING BACTERIA FROM CUCUMBER FERMENTATIONS IN MICHIGAN[a, b, c]

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Lactobacillus plantarum was for some time considered to be the principal species responsible for the acid fermentation of cucumbers. Etchells and Jones *(7)* isolated and studied 49 cultures from commercial fermentations brined at 20, 30 and 40° salometer (5.3, 7.8 and 10.6% NaCl). All 49 cultures were found to be *L. plantarum*. Of 20 cultures isolated from commercial fermentations by Costillow and Fabian *(4)*, 16 were found to be *L. plantarum*.

Recent studies *(12, 13)* of a large number of cultures isolated from fermentations brined in glass jars under laboratory conditions have indicated that there may be up to 5 species of lactic acid bacteria active in cucumber brines. The species found were *Leuconostoc mesenteroides, Streptococcus faecalis, Pediococcus cerevisiae, Lactobacillus brevis* and *L. plantarum* in the order of increasing prevalence. These workers reported that the flora may be influenced by both salt-concentration and temperature of fermentation.

Borg *et al. (1)* identified 92 cultures of acid-forming bacteria from commercial cucumber fermentation; "51 were similar to *Pediococcus cerevisiae*, 28 resembled *L. plantarum* and 13 belonged to another species of *Lactobacillus.*"

This study was initiated to determine the types of acid-forming bacteria active in cucumber fermentations in Michigan. Also, we wished to investigate what effects, if any, that sorbic acid might have on the prevailing types present.

METHODS

Fermentations Studied and Methods of Sampling

Eight hundred and forty-eight cultures of acid-forming bacteria were isolated from 84 cucumber fermentations during the brining seasons of 1953 and 1954. The source of the cultures isolated as to salting stations, fermentations, brining treatments and samples are given in Table 1. The 12 fermentations studied in 1953 were all brined in 45-gal. barrels at one salting station. In 1954, 53 lots were brined in barrels located at 5 salting stations and the other 19 fermentations sampled were commercial lots located at the same 5 stations.

In 1954, one-third of the barrels of each brining treatment at each station were treated with 0.1% sorbic acid, one-third with 0.05% sorbic acid and the other one-third

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[c]Presented before the Fifteenth Annual Meeting of the Institute of Food Technologists, Columbus, Ohio, June 15, 1955.

TABLE 1

Source of acid-forming bacteria

Brining season	Salting station (code)	No. of fermentations	Initial brine concentration[1]	No. of samples	No. of isolations
			Salometer		
Barrel lots:					
1953	M	12	30°	95	380
1954	M	6(dills)	18°	21	33
	M	6	20°	41	97
	M	12	30°	40	47
	M	6	40°	47	90
	NL	6	25°	14	17
	S	6	30°	14	21
	ML	6	30°	17	29
	LT	5	35°	13	21
Commercial tank lots:					
1954	5 stations[2]	19	25–40°	45	113
Totals		84	.....	347	848

[1]The brine strength of all fermentations studied was increased 1° to 2° salometer per week during the active fermentation period except for the 6 dill fermentations.
[2]The 19 commercial scale fermentations were distributed among 5 stations. They were sampled at random at various stages of fermentation.

served as controls. At station LT, 6 barrels were brined but no cultures were isolated from one of them. The per cent sorbic acid was calculated on the basis of the total water in the fermentation; *i.e.*, the water used in making the brine plus 90% of the weight of the cucumbers.

The temperatures of the 1954 brines ranged from 15° to 28°C. with an average of about 20°C.

The brine samples were taken by the technique outlined by Etchells and Jones *(8)* as modified by Costilow and Fabian *(4)*.

Isolation and Identification of Acid-Forming Bacteria

The brine samples were plated by standard procedures on V-8 agar with brom cresol green indicator *(9)*. After 3 days' incubation at 30°C., colonies were picked from the plates of the highest dilutions at which growth occurred. Efforts were made to carry the dilutions high enough so that only 2 or 3 colonies appeared on the plate. The cultures selected were inoculated into trypticase sugar agar (BBL) stabs and refrigerated as soon as visible growth occurred. Monthly transfers were made of the collection and 24-hour subcultures made of stock cultures in micro-inoculum broth (Difco) for inoculating test media.

The bacteria were screened and grouped by determining cell morphology, gram-stain reaction, catalase production, nitrate reduction, reaction in litmus milk and fermentation of dextrose, sucrose and lactose. Catalase tests were run by adding 3% hydrogen peroxide to young cultures in micro-inoculum broth or trypticase sugar agar stabs. Indole-nitrite medium (BBL) was used for nitrate reduction tests and cystine trypticase agar (BBL) was the basal medium for carbohydrate fermentation studies.

On the basis of the screening tests the cultures were broken down into 5 groups as follows: Group A, gram positive cocci similar to *Pediococcus cerevisiae;* Group B, gram positive rods similar to *L. plantarum*; Group C, as Group B except that nitrate was reduced; Group D, similar to *L. brevis*; and Group E, a miscellaneous group of cultures. Over 90% of the cultures were found to belong to the first 4 groups and the remaining cultures were placed in the miscellaneous group. Approximately 10% of each of the first 4 groups and all the cultures in the miscellaneous groups were studied in detail and identified according to the descriptions given in *Bergey's Manual (2)*. Lactobacilli resembling *L. brevis* were tested for gas production by the method recommended by Pederson and Albury *(12)*.

The screening technique used proved to be quite effective in the grouping of these cultures. All cultures in the 4 major groups which were further investigated proved to be constant in reaction. However, two of the major groups were combined when it was found *(6)* that a number of *L. plantarum* strains would reduce nitrate when tested in indole-nitrite medium (BBL). No other consistent difference was found between the groups of this species. A few of the miscellaneous cultures were placed in one or more of the other groupings after more detailed study.

RESULTS

A total of 848 cultures of acid-forming bacteria was obtained from 347 brine samples of 84 cucumber fermentations during the 1953 and 1954 brining seasons. Of the total 848 cultures, 284 (33.5%) were classified as *Pediococcus cerevisiae*, 333 (39.2%) as *L. plantarum*, 188 (22.2%) as *L. brevis* and the remaining 43 (5.1%) comprised a miscellaneous group of bacteria of which one was *Leuconostoc mesenteroides*.

The cultures of *Pediococcus cerevisiae* were very uniform in their morphological and physiological characteristics. All were found to be gram positive cocci with typical tetrad arrangement, catalase positive, nitrate negative, produced no reaction in litmus milk and fermented dextrose but not sucrose or lactose. Twenty-two cultures tested in more detail were, also, quite constant. They were all microaerophilic, had an optimum growth temperature of about 30°C. and did not liquefy gelatin. They all fermented arabinose, dextrose, fructose, maltose, mannose, galactose and salicin. They did not ferment dextrin, inulin, lactose, mannitol, raffinose, rhamnose, sorbitol, starch, sucrose or xylose.

It has been reported recently *(6)* that certain strains of *L. plantarum* will reduce nitrates when tested in semi-solid media. Of the total 333 cultures of this species studied during this work, 173 strains reduced nitrate in indole-nitrite medium (BBL) while 160 strains did not. However, a detailed study of a number of strains failed to reveal any other consistent differences between the two groups.

The classification and distribution of the individual species as to brining seasons and the number of fermentations from which they were isolated are given in Table 2. In 1953, a large percentage of the 380 cultures were obtained during the first 2 weeks of fermentation. This accounts for the relatively high percentage of *Pediococcus cerevisiae* cultures obtained this year; since, as will be shown later, this species predominates during the early period. Also, it should be noted that this species and *L. plantarum*

TABLE 2

Classification and distribution of 848 cultures of acid-forming bacteria

Season	Species	Cultures obtained		Fermentations obtained from	
		No.	%	No.	%
1953	*Pediococcus cerevisiae*	199	52.4	12	100
	L. plantarum	104	27.4	12	100
	L. brevis	65	17.1	11	91.8
	Miscellaneous	12	3.1	6	50.0
1954 (Barrel lots)	*Pediococcus cerevisiae*	63	17.8	28	52.8
	L. plantarum	162	45.6	44	83.0
	L. brevis	111	31.3	32	60.4
	Miscellaneous	19	5.3	16	30.2
1954 (Commercial tanks)	*Pediococcus cerevisiae*	22	19.5	6	31.6
	L. plantarum	67	59.3	17	89.5
	L. brevis	12	10.6	6	31.6
	Miscellaneous	12	10.6	8	42.1
Combined	*Pediococcus cerevisiae*	284	33.5	46	54.8
	L. plantarum	333	39.2	63	75.0
	L. brevis	188	22.2	49	58.3
	Miscellaneous	43	5.1	30	35.7

were isolated from all 12 fermentations in 1953 and *L. brevis* from all but one. More samples were taken from each fermentation in 1953 than in 1954 and, therefore, there was less chance of missing one of the predominating species.

Influence of salt concentration on the acid fermentation. Pederson and Ward *(13)* suggested that salt levels may influence the types of acid-forming bacteria occurring in cucumber fermentations. A comparison of the isolates obtained from 6 fermentations brined at 20° salometer (5.3% NaCl) and from 6 brined at 40° salometer (10.6% NaCl) is given in Table 3. The three major species were isolated in about the same

TABLE 3

Comparison of acid-forming bacteria isolated from fermenting cucumbers brined at 20° and 40° salometer [1]

Species	Brining treatment			
	20° salometer		40° salometer	
	No.	%	No.	%
Pediococcus cerevisiae	17	18.9	14	14.4
L. plantarum	38	42.2	35	36.1
L. brevis	29	32.2	43	44.3
Miscellaneous	6	6.7	5	5.2
Totals	90	100.0	97	100.0

[1] Six barrels of cucumbers were brined according to each treatment.

ratio from both brining treatments. There was a switch in the percentage ratio of *L. plantarum* to *L. brevis* in the higher salometer brines as compared to the lower but it could easily be the result of chance with this small a number of isolations.

Influence of sorbic acid in cucumber fermentations on the incidence of the various species of bacteria. Of the 47 salt stock fermentations in barrels in 1954, 16 were not treated with sorbic acid, 16 were treated with 0.05% sorbic acid and 15 with 0.1% sorbic acid. A comparison of the percentage of isolations from each treatment that each species represented and the percentage of the fermentations of each treatment that the various bacteria were isolated from is presented in Figure 1. It is evident that sorbic acid did not greatly change the acid-forming bacteria flora. However, the percentage of *L. plantarum* isolates decreased with increasing sorbic acid concentrations while that of *L. brevis* increased. This same trend was noted with the percentage of fermentations of each treatment that these species were found in. The frequency of isolation of *Pediococcus cerevisiae* and the miscellaneous group was not appreciably affected.

Estimated sequence of species of acid-forming bacteria in cucumber fermentations. The sequence of the species of acid-forming bacteria was estimated by counting the number of cultures isolated at 5-day intervals during the fermentations and calculating the percentage of the total isolations made at each interval which each species represented. This estimated sequence is given in Figure 2.

Pediococcus cerevisiae was found to be by far the most commonly isolated species during the first 10 days after brining. This fact was, also, shown very clearly in the 1953 studies where of 283 cultures obtained from 12 fermentations during the first 10 days of fermentation, 197 (70%) were of this species. It was noted *(3)* that this was the stage where the highest populations of bacteria occurred in these fermentations and this species was apparently in predominance. However, *Pediococcus cerevisiae* was found very rarely after this period.

L. plantarum and *L. brevis* were isolated the most frequently after the first 10 days of fermentation up to 30 days. At this stage the total populations of acid-forming bacteria were relatively low and not very many cultures were obtained thereafter.

DISCUSSION

It has been shown that three species of lactic acid bacteria are active in cucumber fermentations in Michigan. These are *Pediococcus cerevisiae*, *L. plantarum* and *L. brevis*. These results are in agreement with those of

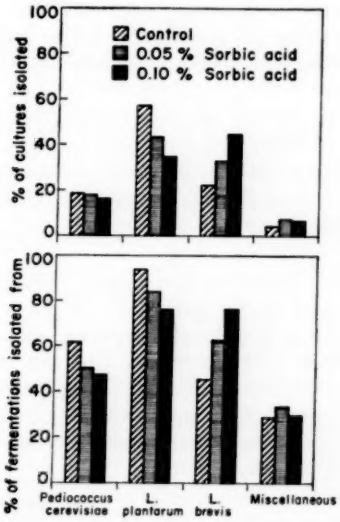

Figure 1. The influence of sorbic acid on the frequency of isolation of the various types of acid-forming bacteria and on the distribution of the cultures among the fermentations studied.

Borg *et al. (1)* and in reasonable agreement with those of Pederson and Ward *(13)* and Pederson and Albury *(12)*. However, these latter workers also found *Leuconostoc mesenteroides* and *Streptococcus faecalis* to be prevalent in many cucumber fermentations. Their data showed that in a number of instances one or both of these species were active while *Pediococcus cerevisiae* was not found. This was particularly true with low salt brines (2.5 to 5%). This difference in our findings may have been due to differences in conditions. The fermentations they studied were in glass

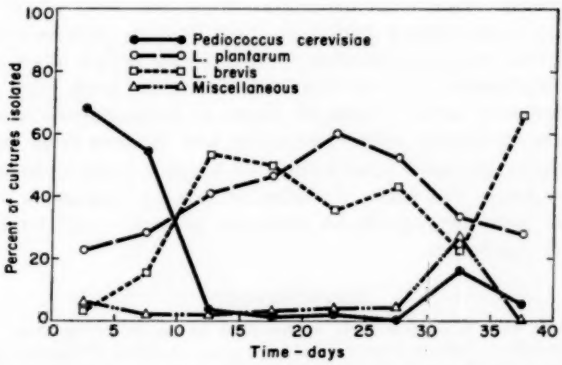

Figure 2. Estimated sequence of the species of acid-forming bacteria in the fermentation of cucumbers.

containers in the laboratory while those studied in this work were in barrel and tank lots under commercial conditions. It has been demonstrated that the microbiological activity in laboratory fermentations may not be comparable with commercial tanks *(4)*. Also, our method of selecting cultures eliminated minor species because only the colonies appearing in the highest dilutions of the brine samples were selected.

No significant effect of salt concentrations ranging from 20° to 40° salometer on the flora occurring in the fermentations was found. This has been reported *(13)* to have a very definite influence on the species found.

As might have been predicted from earlier studies *(5)*, the presence of 0.05 or 0.1% sorbic acid did not have any pronounced effect on the species of lactic acid bacteria isolated from these fermentations. However, there was a trend which indicated that *L. plantarum* was less active in sorbic acid treated fermentations than in the controls, and a greater percentage of *L. brevis* was isolated from the treated lots. It was found in prior studies that *L. plantarum* was apparently more affected by 0.1% sorbic acid than was *L. brevis (5)*.

It should be noted that the cultures of *Pediococcus cerevisiae* isolated in this study were very uniform. They were consistently different from the description of Pederson *(11)* in that no strains fermented sucrose or lactose and all produced catalase. However, Felton *et al. (10)* have demonstrated that this species will produce catalase when grown in some media.

SUMMARY

A study was made of 848 cultures of acid-forming bacteria isolated from 347 brine samples of 84 cucumber fermentations during the 1953 and 1954 brining seasons. These cultures were classified as follows: *Pediococcus cerevisiae*, 284 (33.5%); *Lactobacillus plantarum*, 333 (39.2%); *Lactobacillus brevis*, 188 (22.2%); and miscellaneous, 43 (5.1%).

Pediococcus cerevisiae was the only species which apparently was active at a very definite stage of fermentation. More than 90% of the cultures of this species were isolated during the first 10 days after brining of the cucumbers. This species predominated during this period when the populations of acid-forming bacteria were at their highest level. The other two major species were isolated from all stages of fermentations studied and were the most frequently isolated after the first 10 days from brining.

A comparison of the type of lactic acid bacteria isolated from 20° and 40° salomter brines was made. No effect of brining treatments was noted. Neither was there any significant influence of sorbic acid levels of 0.05 and 0.1% in the brines.

Acknowledgments

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LITERATURE CITED

1. Borg, A. F., Etchells, J. L., and Bell, T. A. The influence of sorbic acid on microbial activity in commercial cucumber fermentations. *Bacteriol. Proc.*, Abst. 19 (1955).
2. Breed, R. S., Murray, E. G. D., and Hitchens, A. P. *Bergey's Manual of Determinative Bacteriology.* 6th ed., 1948, Williams and Wilkins Co., Baltimore, Md.
3. Costilow, R. N., and Coughlin, F. M. (Unpublished data.)
4. Costilow, R. N., and Fabian, F. W. Microbiological studies of cucumber fermentations. *Appl. Microbiol.*, **1**, 314 (1953).
5. Costilow, R. N., Ferguson, W. E., and Ray, S. Sorbic acid as a selective agent in cucumber fermentations. I. Effect of sorbic acid on microorganisms associated with cucumber fermentations. *Appl. Microbiol.*, **3**, 341 (1955).
6. Costilow, R. N., and Humphreys, T. W. Nitrate reduction by certain strains of *Lactobacillus plantarum*. *Science*, **121**, 168 (1955).
7. Etchells, J. L., and Jones, I. D. Characteristics of lactic acid bacteria from commercial cucumber fermentations. *J. Bact.*, **52**, 593 (1946).
8. Etchells, J. L., and Jones, I. D. Procedure for bacteriological examination of brined, salted and pickled vegetables and vegetable products. *Amer. J. Pub. Health*, **36**, 1112 (1946).
9. Fabian, F. W., Fulde, R. C., and Merrick, J. E. A new V-8 medium for determining lactobacilli. *Food Research*, **18**, 280 (1953).
10. Felton, E. A., Evans, J. B., and Niven, C. F., Jr. Production of catalase by the pediococci. *J. Bact.*, **65**, 481 (1953).
11. Pederson, C. S. The genus *Pediococcus*. *Bact. Reviews*, **13**, 225 (1949).
12. Pederson, C. S., and Albury, M. N. Effect of temperature upon bacteriological and chemical changes in fermenting cucumbers. New York State Agr. Expt. Sta., *Bull. 744* (1950).
13. Pederson, C. S., and Ward, L. The effect of salt upon the bacteriological and chemical changes in fermenting cucumbers. New York State Agr. Exp. Sta., *Tech. Bull. 288* (1949).

THE DIGESTION AND ABSORPTION OF FAT[a]

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From 25-40% of the calories in the American diet are normally supplied by fat. In spite of the importance of this single food item, it is only within recent years that a clear understanding has developed of how fat is made available to the body. Digestion and absorption are of particular importance in the case of fats. If these processes occur, then all triglycerides, regardless of the fatty acid composition, are essentially equivalent as sources of calories.

Before reaching the tissues where it is metabolized, fat passes through a number of organs where digestion and absorption could occur. These various organs will be considered in the order in which they come in contact with the fat after it is ingested.

Digestion and Absorption in the Oral Cavity

Fat is not affected by the process of mastication or the added salivary secretion to which food is exposed in the mouth. Thus, in the oral cavity and the esophagus, through which the fat passes shortly after ingestion, there is no digestion or absorption of fat.

Digestion and Absorption in the Stomach

The stomach is the next organ that fat comes in contact with where digestion and absorption could occur. However, the conclusion reached by most experimenters is that fat is little changed during its passage through the stomach. That no hydrolysis occurs is apparently not due to any lack of an enzyme, since Hull and Keaton *(17)* obtained, from an isolated portion of the stomach of a dog, a gastric secretion which possessed lipolytic activity. However, the enzyme was active only in neutral solutions. At the acid pH normally existing in the stomach, the enzyme had little, if any, activity. A recent exception to this concept should be noted in the case of the report of Herting and Ames *(16)* who claim that there is appreciable hydrolysis of trisaturated triglycerides, but not of the more unsaturated triglycerides, in the stomach. However, until it can be established unequivocally that the apparent digestion in the stomach was not due to regurgitation from the intestine into the stomach, this exception must be taken with reservation.

Digestion in the Intestine

From the stomach, the fat, along with other food items, is slowly metered into the small intestine. Shortly after entering the duodenum, the fat comes into intimate contact with the bile from the liver and pancreatic

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juice from the pancreas. It is now that the active phase of fat digestion, followed by absorption, takes place.

Role of bile. The marked surface activity of bile, and the fact that the enzyme responsible for the splitting of fat is water soluble, made for the early, obvious conclusion that the function of bile was to assist in the formation of an emulsion of the fat and water phase so that digestion could take place. Several workers *(21, 24)* have reported that under *in vitro* conditions bile has little effect on the absolute rate of triglyceride hydrolysis. Annegers *(1)*, as well as others, studied the digestion and absorption of fat when bile was diverted from the intestinal tract. It was found that in the absence of bile, triglycerides were extensively hydrolyzed but absorption was quite limited. Thus, the role of bile in the digestive phase is still a moot point. An interesting recent suggestion of Borgström's is that bile shifts the pH maximum of the enzyme lipase so that it more closely coresponds to the pH found in the intestinal tract *(9)*.

Role of pancreatic juice. That pancreatic juice acts upon triglycerides resulting in the release of free fatty acids, was first recognized in 1856 by Claude Bernard. Eighty years passed before the next real advance in this field when, in 1936, Artom *(3)* reported that triglycerides, hydrolyzed *in vitro* by the pancreatic secretion of a dog, yielded products of incomplete saponification. Frazer and Sammons *(14)* later extended these studies and established monoglycerides as one of the hydrolysis products of triglycerides. It remained, however, for Desnuelle *et al. (11)* to show unequivocally that the hydrolysis of triglycerides was a series of stepwise reactions from triglyceride to diglyceride to monoglyceride to glycerol with fatty acid being released at each step. The quantitative aspects of this work were weakened by the supposition that the hydrolysis was a random one.

During the same period studies were being carried out showing that similar products were formed in living animals. Thus, the high hydroxyl value of the lipides isolated from the lumen of the intestinal tract of rats was interpreted by Frazer and Sammons *(14)* as showing the presence of monoglycerides. Although no experimental details were given, a later paper stated that monoglycerides were found in the intestinal tract of human subjects *(13)*. In 1952 Kuhrt *et al. (19)* reported that lipides recovered from the intestinal tract contained extremely large amounts of monoglycerides.

The final sequence in this series of events was the report from our laboratories on the hydrolysis products of triglycerides *(22)*. The earlier work had demonstrated that the hydrolysis of triglycerides was a series of stepwise reactions, but no consideration had been given to the isomeric form of the mono- and diglycerides formed. The diglycerides could be either the 1, 3- or 1, 2-isomer, while the monoglycerides could be either the 1- or 2-isomer. Any, or all, of these isomers could be formed depending upon the nature of the reaction. Because of the lack of suitable techniques up to that time, it was impossible to distinguish between these various isomers. The demonstration by J. B. Martin *(20)* that 2-monoglyceride could be quantitatively converted to the 1-isomer, made possible the determination of this question.

Table 1 gives the composition of the lipides recovered from the stomach and small intestine of a rat, 3 hours following the feeding of a triglyceride.

TABLE 1

The composition of the lipides recovered from the lumen of the stomach and intestine of rats three hours following the feeding of triglyceride (22)

	Stomach	Intestine
	Wt. %	Wt. %
Triglyceride	....	41
Diglyceride	....	36
Monoglyceride	1.4	7.5
1-isomer	1.4	3.2
2-isomer	0.	4.3
Free fatty acid	4.8	15.5

It will be noted that little or no free fatty acids or monoglycerides were found in the stomach, once again showing that little digestion of fat takes place in the stomach. However, in the intestinal tract appreciable amounts of digestion products, free fatty acids, monoglycerides, and diglycerides, are present. The monoglycerides found were about equally divided between the 1- and 2-isomer.

The finding that both 1- and 2-monoglycerides were present in the intestinal lipides gave no indication as to the specific route of hydrolysis that was being followed. The route, however, could be established by the feeding of a triglyceride in which the fatty acids were on known positions of the glyceride molecule. For this purpose rats were administered 2-oleoyl dipalmitin. Three hours later the lipides were recovered from the lumen of the intestinal tract, the monoglycerides and free fatty acids isolated, and the nature of their fatty acids determined. From these data it appeared that the monoglycerides were predominantly monoolein and the free fatty acids were palmitic acid. Hydrolysis then appeared to be limited to the fatty acids on the 1 and 3 position of glycerol.

Since, in the lipides of the intestinal tract, both 1- and 2-monoolein were present, the 2-monoolein must be the immediate hydrolysis product that was formed and the 1-monoolein arose as a result of isomerization of the 2-monoolein. Since it is the 2-monoglyceride that is formed, the intermediate between the triglyceride and the monoglyceride is likely to be the 1,2-diglyceride. The probable route of hydrolysis of triglycerides, which would be in agreement with the results of these studies, is hydrolysis of triglyceride to 1,2-diglyceride to 2-monoglyceride, with fatty acid being released at each step.

The formation of 2-monoglycerides and 1,2-diglycerides was subsequently confirmed by Borgström *(8)*. More recently, Harris *et al. (15)* and Blankenhorn and Ahrens *(4)* have demonstrated the formation of 2-monoglycerides and 1,2-diglycerides in the intestinal tract of human subjects.

Studies using living animals have the disadvantage that there is continual addition of new triglycerides from the stomach and absorption of the digestion products through the intestinal wall. For this reason, studies *in vitro* have definite advantages for characterizing reactions. Using such conditions, Borgström was able to demonstrate that 1,2-diglycerides and 2-monoglycerides are formed during the hydrolysis *in vitro* of triglyceride by pancreatic lipase. This he has been able to demonstrate by actual

isolation and chemical determination of the end products *(8)* and by an ingenious use of radio-active isotopes *(10)*. Since lipase is an esterifying as well as hydrolyzing enzyme, he incubated a mixture of untagged triglycerides and radio-active tagged fatty acids in the presence of pancreatic lipase. At the end of the digestion period, the mono-, di-, and triglycerides were isolated and the amount of tagged fatty acid, which had been incorporated into the various glycerides, was determined. Based on the ratio of tagged fatty acids among the various types of glycerides, it appeared that only the 1 and 3 positions of glycerol participated in the reactions.

The studies in our laboratories have been extended to an investigation of the hydrolysis of triglycerides *in vitro (21)*. Figure 1 shows the time-course reaction curves that are obtained under these particular conditions.

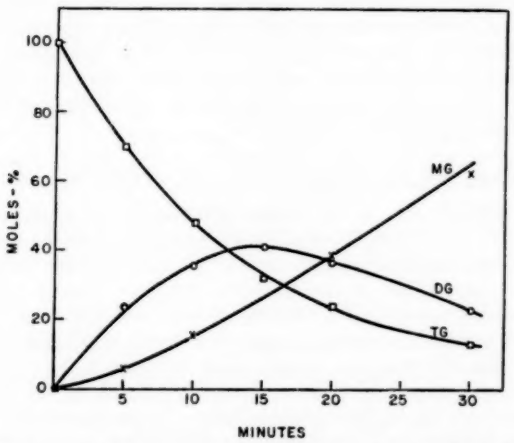

Figure 1. The course of the enzymatic hydrolysis in vitro of triglycerides. Digestion conditions: 13.6 ml. of 1M ammonium chloride/ammonium hydroxide buffer, pH 8.0; 0.3 ml. of 0.15% solution of bile salts; 1.0 ml. of 45% calcium chloride solution; 2.3 ml. of aqueous suspension of 156 mgm. steapsin; 2.0 g. of cottonseed oil; pH of 8.0 maintained by addition of 4M ammonium hydroxide; temperature 40 ± 0.5°C. (21).

The glyceride curves obtained are the type that would be expected for a series of consecutive reactions. Apparently the monoglyceride is the final digestion product under these conditions in that no free glycerol was present, even after 30 minutes' hydrolysis. Analyses of these curves show the conversion of triglyceride to diglyceride to be a pseudomonomolecular reaction. However, the conversion of diglyceride to monoglyceride is indicated to be a zero order reaction. Such characterization of these reactions would have been impossible within the animal organism.

The establishment of the diglyceride and monoglyceride isomers resulting when triglycerides are hydrolyzed *in vitro* was carried out using techniques similar to those used with the animals. Triglycerides in which the position of the fatty acids on the glycerol molecule was known were hydrolyzed with pancreatic lipase. The digestion products were isolated and characterized in order to establish their structure. The results of such an

TABLE 2

Iodine values of the enzymatic hydrolysis products of 2-oleoyl dipalmitin (21)

Hydrolysis products	Values for theoretical products			Found
	Random	1,3-DG→1-MG	1,2-DG→2-MG	
Triglycerides	29	29	29	30
Diglycerides	27	0	43	44
Monoglycerides	24	0	71	61
Free fatty acids	30	45-90	0	8

experiment, when 2-oleoyl dipalmitin was used as the substrate, are given in Table 2. From these data it appears that the diglyceride formed was oleoyl palmitin and the monoglyceride was monoolein.

Thus, the course of hydrolysis *in vitro*, as well as *in vivo*, is from triglycerides to 1,2-diglyceride to 2-monoglyceride with fatty acid being released at each step. Moreover, there is little or no hydrolysis of the fatty acid located on the 2-position of the glycerol. From these results, it appears that the enzyme lipase is specific for reactions occurring at the primary hydroxyl group of glycerol.

TRANSPORT OF LIPIDES FROM THE INTESTINAL WALL

The next logical step in this discussion would be consideration of fat absorption. However, before considering this subject, it will be worthwhile to consider the nature of the lipides, leaving the intestinal tract following the completion of the absorptive stage. Having established the digestion products formed in the lumen of the intestinal tract, and knowing the nature of the lipides being transported from the intestinal tract, one can estimate the changes that lipides must undergo in the intestinal wall.

There are two routes by which absorbed fat can be transported from the intestinal tract to the rest of the body. These routes are the thoracic duct and the portal vein. The technique of Bollman *et al. (7)* for cannulating the thoracic duct of rats, has been of great help in obtaining information about the nature of the lipides in the thoracic duct.

Since it was established that partial glycerides and free fatty acids are formed in the intestinal tract as a result of the digestive phase, it was of interest to determine the nature of the lipides leaving the intestinal wall. In 1936, Artom *(2)* had demonstrated that following the feeding of triglycerides, only triglycerides could be found in the thoracic duct lymph of dogs. Recently these studies were extended in our laboratory using rats as the test animals *(23)*. The rats were fed diets containing 25% of free fatty acid, monoglyceride, diglyceride, or triglyceride. The thoracic duct was cannulated and the lymph of the animals collected and analyzed for free fatty acid and monoglyceride content. Regardless of the nature of the lipides that were fed to the animals essentially no monoglycerides or free fatty acids could be found in the thoracic duct lymph. Thus, one of the processes that takes place in the intestinal wall is a resynthesis of triglycerides from the digestion products formed in the intestinal lumen.

Experiments carried out in the laboratory of Chaikoff have employed this cannulation technique combined with sampling of venous blood in order to demonstrate the route by which fatty acids leave the intestinal

TABLE 3

Per cent of fatty acids of various chain lengths transported by the thoracic duct (5, 6, 18)

Chain length	% in thoracic duct
18	95
16	97
14	70
12	35
10	10

tract. It will be noted that a fatty acid conaining 12 carbon atoms is transported partly by way of the thoracic duct and partly by way of the portal vein. The more water-soluble fatty acids leave the intestinal tract by way of the portal vein, whereas the more fat-soluble fatty acids are transported by way of the thoracic duct. A similar distribution of fatty acids was found by Fernandes *(12)* in a child with chylothorax. Thus, another process that is taking place in the intestinal wall is the segregation of the fat and water soluble fatty acids into their respective channels for transport.

Recently the digestion and absorption of the triglyceride, stearoyl diacetin, has been studied in our laboratory. Although these experiments are not as yet complete, the results thus far obtained indicate that the stearic acid portion of the molecule is partially converted to palmitic acid and unsaturated fatty acids by the time it reaches the thoracic duct. Thus, another process that is going on in the intestinal wall is the changing of the amount of saturation and the chain length of the absorbed fatty acids.

ABSORPTION FROM THE INTESTINAL TRACT

From the above studies a relatively clear picture of the mechanism of fat digestion is now available. The same cannot be said of fat absorption. Over the period of years two theories of fat absorption have developed. According to the hydrolytic theory, fat is completely hydrolyzed in the intestinal tract to free fatty acids and glycerol. The fatty acids form an emulsion with the bile and in this form they are absorbed. The particulate theory, on the other hand, holds that digestion in the intestinal tract proceeds only far enough to yield sufficient monoglycerides and free fatty acids, which together with the bile salts, emulsify the remaining triglycerides. The main bulk of the fat is absorbed in the form of triglycerides.

The interesting thing about the two theories is that they involve reactions and processes occurring in a single layer of cells. Actually, better than 90% of the intestinal tract is purely supportive and only the remaining small fraction is concerned with absorption; this is a layer of epithelial cells of single cell thickness. The problem is one of transportation of fat or its digestion products from the lumen of the intestinal tract to the interior of this epithelial cell and discharging the fat on the other side of the cell where it is picked up by the intestinal lymphatics. However, the simple absorption and transport of fat across a single cell are not the only processes going on. As noted above, there is resynthesis of triglycerides, changing the saturation of the fatty acid, changing the chain length of the fatty acid, and the directing of the water-soluble fatty acids to the portal vein and the fat-soluble fatty acids to the thoracic duct. Until a

better understanding is obtained of these various processes that are going on in the intestinal wall, the results obtained in any experiment which is designed to study the absorption of fat is bound to be obscure.

One of the most important questions that remains to be answered in this field is whether the resynthesis of triglycerides in the intestinal wall is a directed or random one. Practically all of the experimental results reported thus far on the absorption of fat have assumed that the resynthesis of triglycerides is a random one. If, however, it should be determined that the synthetic process is a directed one, as is the case in the digestive process, these experiments on the mechanism of absorption will have to be re-evaluated. At present, one must conclude that the mechanism of fat absorption has not been established. However, a number of laboratories are currently working on this problem and one can anticipate that within a few years a final definitive answer to this problem will be attained.

LITERATURE CITED

1. ANNEGERS, J. H. Function of pancreatic juice and of bile in assimilation of dietary triglyceride. *Arch. Int. Med.*, **93**, 9-22 (1954).
2. ARTOM, C., AND PERETTI, G. The lipides of the lymph of the thoracic duct of dogs during the absorption of neutral fats. *Boll. soc. ital., biol. sper.*, 10, 877-880 (1935).
3. ARTOM, C., AND REALE, L. The formation of intermediate products in the pancreatic digestion of neutral fat. *Boll. soc. ital., biol. sper.*, 10, 883-884 (1935).
4. BLANKENHORN, D. H., AND AHRENS, E. H. Extraction, isolation, and identification of hydrolytic products of triglyceride digestion in man. *J. Biol. Chem.*, **212**, 69-81 (1955).
5. BLOOM, B., CHAIKOFF, I. L., AND REINHARDT, W. O. Intestinal lymph as pathway for transport of absorbed fatty acids of different chain lengths. *Am. J. Physiol.*, **166**, 451-455 (1951).
6. BLOOM, B., CHAIKOFF, I. L., REINHARDT, W. O., ENTENMAN, C., AND DAUBEN, W. G. The quantitative significance of the lymphatic pathway in transport of absorbed fatty acids. *J. Biol. Chem.*, **184**, 1-8 (1950).
7. BOLLMAN, J. L., CAIN, J. C., AND GRINDLAY, J. H. Technique for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. *J. Lab. Clin. Med.*, **33**, 1349-1352 (1948).
8. BORGSTRÖM, B. On the mechanism of the hydrolysis of glycerides by pancreatic lipase. *Acta Chem. Scand.*, **7**, 557-558 (1953).
9. BORGSTRÖM, B. Effect of tauro-cholic acid on the pH/activity curve of rat pancreatic lipase. *Biochim. et Biophys. Acta*, **13**, 149-150 (1954).
10. BORGSTRÖM, B. On the mechanism of pancreatic lipolysis of glycerides. *Biochim. et Biophys. Acta*, 13, 491-504 (1954).
11. DESNUELLE, P., NAUDET, M., AND ROUZIER, J. Quantitative studies on the partial, enzymatic hydrolysis of triglycerides. *Biochim. et Biophys. Acta*, **2**, 561-574 (1948).
12. FERNANDES, J. Fat absorption studied in a child with chylothorax. Thesis, Univ. Leyden, pp. 76 (1953). From *Nutr. Abst. Rev.*, **24**, 609 (1954).
13. FRAZER, A. C. Aetiology of steatorrhea. *Brit. Med. J.*, **2**, 641-645 (1947).
14. FRAZER, A. C., AND SAMMONS, H. G. The formation of mono- and di-glycerides during the hydrolysis of triglyceride by pancreatic lipase. *Biochem. J.*, **39**, 122-128 (1945).
15. HARRIS, R. S., CHAMBERLAIN, J. W., AND BENEDICT, J. H. Formation of monoglycerides during fat digestion in human beings. *Fed. Proc.*, **13**, 525 (1954).
16. HERTING, D. C., AND AMES, S. R. On the gastric lipolysis of fat. *Arch. Biochem. Biophys.*, **55**, 295-297 (1955).
17. HULL, M., AND KEATON, R. W. The existence of a gastric lipase. *J. Biol. Chem.*, **32**, 127-140 (1917).

18. KIYASU, J. Y., BLOOM, B., AND CHAIKOFF, I. L. The portal transport of absorbed fatty acids. *J. Biol. Chem.*, **199**, 415-419 (1952).
19. KUHRT, N. H., WELCH, E. A., AND NASSET, E. S. Isolation and identification of monoglycerides in the intestinal contents of humans. *J. Am. Oil Chem. Soc.*, **29**, 271-278 (1952).
20. MARTIN, J. B. The equilibrium between symmetrical and unsymmetrical monoglycerides and determination of total monoglycerides. *J. Am. Chem. Soc.*, **75**, 5483-5486 (1953).
21. MATTSON, F. H., AND BECK, L. W. The digestion in vitro of triglycerides by pancreatic lipase. *J. Biol. Chem.*, **214**, 115-125 (1955).
22. MATTSON, F. H., BENEDICT, J. H., MARTIN, J. B., AND BECK, L. W. Intermediates formed during the digestion of triglycerides. *J. Nutrition*, **48**, 335-344 (1952).
23. RELLER, H. H., BENEDICT, J. H., MATTSON, F. H., AND BECK, L. W. Composition of lymph lipids in rats fed fat digestion products. *Fed. Proc.*, **13**, 474 (1954).
24. WILLS, E. D. The effect of anionic detergents and some related compounds on enzymes. *Biochem. J.*, **57**, 109-120 (1954).

FREEZING, PACKAGING AND FROZEN STORAGE OF FISH

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Problems of freezing and frozen storage of fish are very much like those for other frozen foods such as meat and poultry. During cold storage these products undergo changes of two general types, chemical and physical. Chemical changes include those brought about by the action of bacteria, by naturally occurring enzymes, by oxidative and hydrolytic processes in the fats and oils, and by denaturation of proteins. The principal physical changes are ice crystal formation and desiccation or drying out of the flesh.

The main effect of freezing and frozen storage on the quality of fish is a change in texture which is connected with the accompanying rearrangement and coagulation of the proteins in the muscle. The tissues become tough because of the denaturation of the proteins. The present investigators *(6)* have shown that thawing and refreezing also alter the state of the muscle proteins (myosin), making them more liable to undergo denaturation during frozen storage, defrosting, and subsequent storage.

The theory of quick freezing is based on the observation that there is a critical zone at about −4 to −10°C. in which rapid denaturation occurs *(3)*; this temperature zone should be passed as rapidly as possible during the freezing process. However, the rate of freezing is not as important as it was considered to be formerly *(2, 9, 10, 11)*. Reay *(10)* concludes that the freshness of fish and the temperature of storage are more important, the rate of freezing being a minor factor. On the other hand, the present investigators *(6)* have found that freezing of fish during *rigor mortis* makes myosin more prone to undergo denaturation during cold storage and defrosting than when the fish are frozen after the resolution of *rigor mortis*. This conclusion is confirmed by Marsh *(5)*, who found that freezing of meat before *rigor mortis* is completed can lead to abnormal behavior on thawing. Under all circumstances there is a definite change in some characteristics of the fresh fish after freezing and subsequent thawing, even if we must admit that the changes occurring during storage are more serious.

This paper deals with problems concerning the quality of products that arise in the cutting, filleting, packaging, freezing, and frozen storage of fish.

METHODS

Baltic herring *(Clupea harengus* var. *membranus)* was used in this study. Fish or fillets packed in waxed cartons were frozen in a freezer with forced air circulation or in a metal contact freezer at −35°C. The frozen fish were stored at −20°C., defrosted after suitable intervals in pliofilm bags during 7 hours at room temperature, and then examined for quality. The quality of the fish was estimated by determining the myosin denaturation using the procedure described previously by the authors *(6, 7)*. The details of preparing the fish, packaging, etc. are reported in connection with the results.

RESULTS

The curves in Figure 1 show the myosin denaturation in the fish frozen whole and stored 10 months at —20°C. The glazed and the unglazed fish were stored in cold rooms with and without air circulation. The glazed fish remained in good condition for a longer time than did the unglazed fish in both types of storage. The glazed fish kept in the storage without air circulation began to undergo denaturation after 8 months, but the fish kept in the cold room with air circulation began to deteriorate after 5 months. Thus, mechanical air circulation was shown to have a detrimental effect on the quality of frozen fish. The determinations of dry-matter in fish from all 4 series showed that no desiccation or drying out took place in the fish during frozen storage. The ice layer on the surface of glazed fish evaporated slowly, however. The loss of quality was attributed to the denaturation of the proteins.

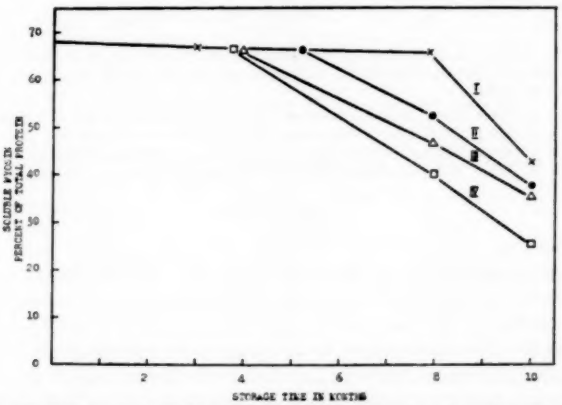

Figure 1. Variation of solubility of myosin from glazed and unglazed fish stored at —20°C. in cold rooms with and without air circulation.

I—glazed fish, storeroom without air circulation.
II—glazed fish, storeroom with air circulation.
III—unglazed fish, storeroom without air circulation.
IV—unglazed fish, storeroom with air circulation.

The experiments illustrated in Figure 2 were carried out with glazed whole fish packed in waxed one-kilogram cartons. Twenty cartons were packed together (a) in cases lined with thin aluminum foil or (b) in unlined cases. The frozen fish was stored in the same storerooms as described. The results show that the packaging of fish in air-tight cases has eliminated the detrimental effect of air circulation. The quality of frozen fish decreased to about the same degree in all 4 series.

The results obtained in the experiment carried out with fillets and whole fish frozen in an air blast and whole fish frozen in a contact freezer are summarized in Figure 3. The curves show that the fish frozen in the air blast freezer began to undergo denaturation after 4 months, but the fish frozen in the contact freezer about 2½ months later. This indicates that contact freezing yields a better quality of fish than air blast freezing. The fish fillets and whole fish frozen in the air blast deteriorate in quality at approximately the same rate.

The curves in Figure 4 show the brine solubility of myosin when fish is frozen before and after *rigor mortis*. In the former case the decrease in myosin extractability starts very early and is very pronounced. It will be noted that the extractability of myosin has decreased by 42% about eight hours after freezing. In contrast, the fish frozen after the resolution of *rigor mortis* has remained in its original state during the experiments. Thus, avoiding the freezing of *pre-rigor* fish is of critical importance in preserving the quality of the frozen product.

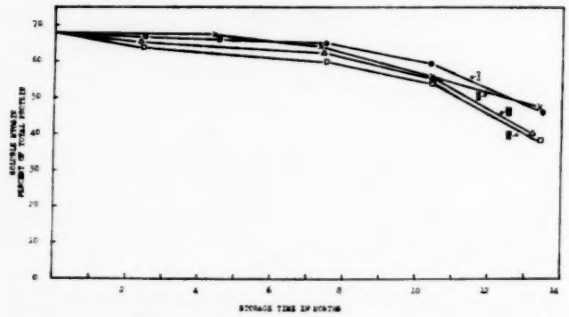

Figure 2. Variation of solubility of myosin from glazed fish in waxed cartons packed into cases with and without aluminium foil were stored at —20°C. in cold rooms with and without air circulation.

I—fish in cases lined with aluminium foil, storeroom with air circulation.
II—fish in cases lined with aluminium foil, storeroom without air circulation.
III—fish in cases without aluminium foil lining, storeroom without air circulation.
IV—fish in cases without aluminium foil lining, storeroom with air circulation.

DISCUSSION

Considerable work has been done on the effect of the rate of freezing and storage temperature on the quality of fish. The findings have proved that the freezing rate and size of the ice crystals are of lesser importance from the viewpoint of the quality of the frozen fish when freezing is reasonably rapid. The dimensions of the small ice crystals formed during extremely rapid freezing increase gradually during storage. Fluctuation of the storage temperature is particularly conducive to the formation of large ice crystals. Thus the advantage of quick freezing is eliminated to a large

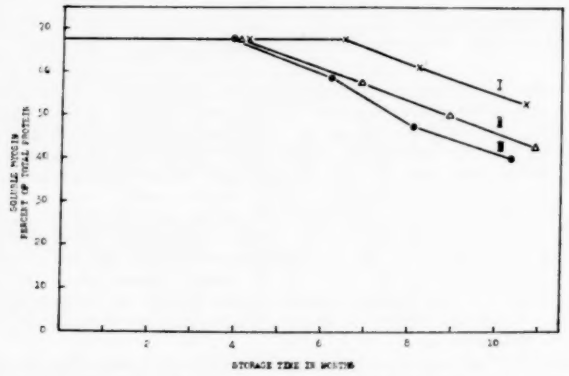

Figure 3. Variation of solubility of myosin from fish frozen in air blast and in contact freezer and stored at —20°C.

I—whole fish frozen in contact freezer.
II—fillets frozen in air blast.
III—whole fish frozen in air blast.

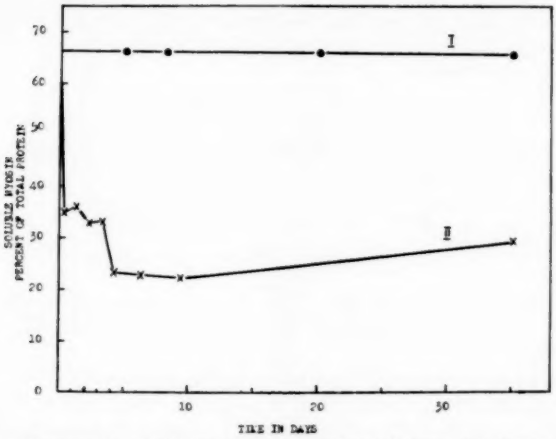

Figure 4. Decrease in solubility of myosin extracted from fish frozen before and after rigor mortis and stored at —20°C.

I—post-rigor fish.
II—pre-rigor fish.

degree. Most of the adverse changes sometimes encountered in frozen fish result not from any changes brought about by the freezing process itself, but rather through changes that occur during the storage of the frozen products. One such change is the denaturation of protein which leads to a drier and more fibrous product. Other changes, such as dehydration and oxidation, seem to be of less importance for lean fish, and may be prevented by moisture-proof packages and by glazing. When fish are stored with only an ice glaze, the humidity of the storage space is of prime importance, and unless steps are taken to keep the atmosphere in the room saturated, the glaze will evaporate quickly and thereby permit deterioration due to dehydration. The temperature in the storage room should not be allowed to fluctuate, and other conditions should be held constant. These aspects are in good accordance with the findings in the present experiments.

Since changes in the consistency of the fish flesh are related to the state of the proteins in the muscle, much work has been done to characterize these proteins, Hamoir *(4)*, Connell *(1)*, Nikkilä and Linko *(8)*. It has been found, for instance, that protein denaturation during frozen storage and defrosting is confined to the fraction, myosin, which in time becomes completely insoluble *(6)*. This furnishes the technologist with a chemical method of estimating the loss in quality of frozen fish. The storage temperature has by far the greatest effect on myosin denaturation. Hence, the question of the best storage temperature is only a question of keeping the temperature as low as possible. On the other hand, the present results show that the preparation and packaging of fish and the type of freezer also have a pronounced effect on the quality of frozen products. Surface dehydration and mechanical air circulation during storage may lead to rapid myosin denaturation. The investigators have shown previously *(6)*

that refreezing effects a marked denaturation, which means that fish which have become defrosted, *e.g.* during transport, can no longer be refrozen with satisfactory results.

Use of fresh fish for freezing is important. However, fish must not be frozen before or during *rigor mortis*, because in this stage myosin undergoes very extensive change as shown by the decrease in extractability (Figure 4 and reference *6*).

SUMMARY AND CONCLUSIONS

The factors which have an important influence on the quality of frozen fish have been considered.

Proper wrapping and careful glazing protect the fish against atmospheric influences. Air spaces within the package should be kept at a minimum. Quick freezing under pressure in a contact-type freezer is superior to freezing in an air blast. The temperature of freezing storage should be held constant at all times, and should not be permitted to fluctuate over a wide range. Mechanical air circulation in the storeroom is detrimental. Fish intended for freezing must be fresh for best results with respect to quality, but should have passed the stage of *rigor mortis*.

LITERATURE CITED

1. Connell, J. J. Studies on the proteins of fish skeletal muscle. 2. Electrophoretic analysis of low ionic strength extracts of several species of fish. *Biochem. J.*, **55**, 378 (1953).
2. Dyer, W. J. Protein denaturation in frozen and stored fish. *Food Research*, **16**, 522 (1951).
3. Dyer, W. J. *Proceedings, Symposium on Cured and Frozen Fish Technology.* 1953, SIK-Publication No. 100, Göteborg, Sweden.
4. Hamoir, G. *Proceedings, Symposium on Cured and Frozen Fish Technology.* 1953, SIK-Publication No. 100, Göteborg, Sweden.
5. Marsh, B. B. *Rigor mortis* in beef. *J. Sci. Food Agr.*, **5**, 70 (1954).
6. Nikkilä, O. E., and Linko, R. R. Denaturation of myosin during defrosting of frozen fish. *Food Research*, **19**, 200 (1954).
7. Nikkilä, O. E., and Linko, R. R. Denaturation of myosin in fish muscle and in myosin sol by salts. *Suomen Kemistilehti*, B **27**, 37 (1954).
8. Nikkilä, O. E., and Linko, R. R. Paper electrophoretic analysis of low ionic strength protein extracts from fish skeletal muscle. *Biochem. J.*, **60**, 242 (1955).
9. Notevarp, O., and Heen, E. The influence of freezing rate, storage temperature and freshness of raw material on the quality of frozen fish. *Z. ges. Kälte-Ind.*, **47**, 122 (1940).
10. Reay, G. A. The freezing and cold storage of white fish. *Rept. Food Investigations Board*, Great Britain. (1939), **43** (1940).
11. Young, O. C. The quality of fresh frozen and stored halibut as determined by a tasting panel. *J. Fisheries Research Board*, Can., **37**, 12 (1938).

SOME FACTORS AFFECTING GROWTH OF *BACILLUS COAGULANS* IN TOMATO JUICE[a]

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Since the discovery that bacteria can cause serious losses to commercial packers of tomato products, much work has been done to find adequate methods for controlling this spoilage. Most of these studies have been concerned with the factors which will inhibit spore germination and growth, or factors which will affect the heat resistance of the spoilage organisms.

Pederson and Becker *(8)* and Becker and Pederson *(1)* from an extensive study of *Bacillus coagulans (Bacillus thermoacidurans)* and its ability to spoil tomato juice, concluded that pH was the limiting factor for growth of the bacterium. They offered this as an explanation for the sporadic spoilage of tomato juice as they observed variations in the pH of different lots of tomato juice produced in the same area. Rice and Pederson *(10)* studied the effects of variations in concentrations of spores of *B. coagulans* on spoilage and found that as the size of the inoculum decreased, the minimum pH at which growth occurred increased.

In view of the important bearing that environmental factors have on spores, studies were initiated to determine some effects of environment on germination.

METHODS AND MATERIALS

Cultures. The cultures used in these studies were strains of *B. thermoacidurans.* All were reported as having been originally isolated from spoiled canned tomato products and were obtained as follows: F.S. 700, F.S. 744, and F.S. 787, Continental Can Co.; F. 4, American Can Co.; and B 1175 and B 1187, Bureau of Agricultural and Industrial Chemistry, U.S.D.A., Peoria, Ill. Morphological, cultural, and biochemical studies of these strains were in close agreement with the description given for *B. coagulans* (Hammer) by Breed, Murray, and Hitchins *(2)* and Gordon and Smith *(4).*

Preparation of inoculum. Young cultures of vegetative cells were kept available by daily transfers to fresh thermoacidurans (T.A.)[c] broth (pH 6.0). These cultures were presumed to be free of spores as frequent spore stains were negative.

Spore suspensions were prepared by growing the strains on the surface of T.A. Agar (Difco) for 5 days at 50°C. in Roux flasks, and aseptically harvested with phosphate buffer (pH 7.0). After washing the suspensions three times with phosphate buffer by centrifugation, half of each suspension was stored at 2°-4°C.; the other half was heated rapidly in a water bath and held at 100°C. for one minute and cooled rapidly before storage. The spore suspensions were counted by serial dilution techniques using T.A. Agar (Difco) and incubation for 48 hours at 50°C. Routinely, approximately 20,000 spores were used for inoculum.

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[c]

Proteose peptone	0.5%
Yeast Extract	0.5%
Glucose	0.5%
KH_2PO_4	0.4%

Plating techniques. The number of viable cells in an environment was determined either turbimetrically or by dilution plating methods. T.A. broth and Agar (Difco) were used as the culture medium. Incubation temperatures varied with the experiment, but were usually at 50°C.

Source of tomato juice. U.S. Grade "A" tomato juice, obtained from a local processor, was used throughout this investigation. The acidity varied from pH 4.25 to 4.30.

Hydrogen-ion concentration measurements. All pH determinations were made using a Beckman Model H-2 glass electrode pH meter. The various substrates were adjusted to the desired pH using KOH and HCl.

RESULTS

Aliquots of juice (pH 4.27), sterilized for 15 minutes at 121°C. were inoculated with both stored spores and vegetative cells of the different strains and incubated at 37°C. and 50°C. After 5 days incubation, no changes could be noted in the tomato juice and the pH had not changed significantly. This indicated that the strains had lost their ability or could not spoil tomato juice at the above conditions.

Strains of *B. thermoacidurans* are known to change in physiology and to lose their ability to spoil tomato juice (Becker and Pederson, *1*). Tomato juice serum (the clear, supernatant fluid obtained by filtering tomato juice) was adjusted to pH 5.0, sterilized, and inoculated with vegetative cells of the different strains. After 24 hours incubation at 37°C. and 50°C. good growth was noted in all cases. By progressively lowering the pH of the tomato juice serum, all strains were adapted to grow at pH 4.2 and in some instances lower.

The ability of the different strains to spoil tomato juice was again tested. Tomato juice (pH 4.26) was inoculated with vegetative cells growing in tomato juice serum (pH 4.2). In all cases, after 5 days' incubation, the tomato juice had the typical characteristics of flat-sour spoilage.

Cultural characteristics. Optimum conditions for growth, in relation to the temperature of incubation and the pH of the environment, were determined by inoculating vegetative cells in TA broth adjusted to varying hydrogen-ion concentrations (pH 4.5 to pH 9.0) and incubating at temperatures varying from 20°C. to 65°C. Growth was determined turbimetrically after 24 hours incubation. Good growth was obtained from 30°C. to 55°C. and from pH 5.0 to pH 7.5. Little difference could be noted at levels between pH 5.5 and pH 7.0 or at temperatures between 35°C. and 55°C.

Stored spores of the different strains were tested for their ability to germinate and grow, using varying conditions of pH and temperatures. Spores of all strains germinated and grew in TA broth at pH 5.0. In no case was growth observed at pH 4.3. Growth was obtained when some of the inoculated broth of pH 4.3 was transferred to fresh broth of pH 5 and incubated at 45°C.

Effects of potassium phosphate on spore germination. In attempts to force germination with subsequent growth at pH levels comparable to those found in tomato juice, different concentrations of various constituents of TA broth were used. It was noted that as the concentration of the KH_2PO_4 was increased, the time required for visible growth decreased.

TA broth (pH 6.0), containing progressively increasing quantities of KH_2PO_4 was inoculated with both stored spores and vegetative cells of F.S. 787. Incubation was at 45°C. Viable cell counts, taken at periodic intervals, showed that the concentration of KH_2PO_4 affected the lag-phase of growth when spores were used for inoculum but had little effect when vegetative cells were used. Growth, when spores were used for inoculum, was not noted until 0.025 M. KH_2PO_4 was incorporated in the medium. At this concentration the lag-phase was 19 hours and became progressively shorter until it reached 11 hours when 0.15 M. KH_2PO_4 was used (Figure 1). The concentration of KH_2PO_4 seemed to have no effect on the lag-phase when vegetative cells were used for inoculum as it remained rather constant at seven hours (Figure 2).

In a series of similar experiments, in which Na_2HPO_4, NaCl, and KCl in concentrations comparable to those used in the previous experiment, replaced the KH_2PO_4 in TA broth, the spores appeared to germinate and grow only when phosphate was used (Figure 3). These salts had little effect on the lag-phase when vegetative cells were used for inoculum (Figure 4).

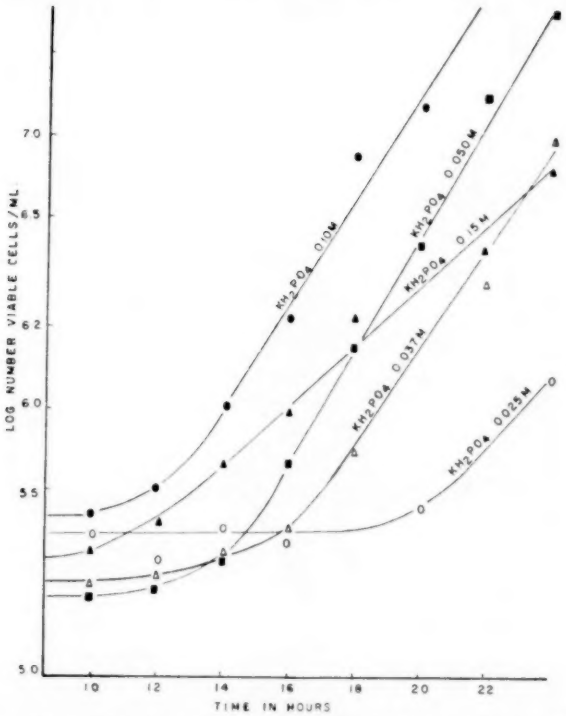

Figure 1. Effects of potassium phosphate concentration on the growth curve of B. thermoacidurans F.S. 787 using spores for inoculum.

The spoilage of tomato juice by spores. Tomato juice, with 0.05M, KH_2PO_4 added and adjusted with HCl to its original acidity (pH 4.26), was inoculated with stored spores of the various strains and incubated for 5 days at 37°C. and 50°C. In all cases the juice had the characteristics of flat-sour spoilage and the titrable acidity had increased.

Aliquots of the harvested cell suspensions, which had been stored, were heated (100°C. for one minute) and inoculated into tomato juice (pH 4.26) immediately after heating. As a control the stored spores were again tested for their ability to spoil tomato juice. After 5 days' incubation, characteristic flat-sour spoilage was noted only in the tomato juice that had been inoculated with the freshly heat-treated spores.

DISCUSSION

The results of these studies show that spores of *B. coagulans* prepared in the usual manner by heating harvested cell suspensions to 100°C. are capable of germinating and growing in tomato juice only if they are inoculated immediately after the heat treatment. These results can be explained on the basis of germination of heat activated spores described by Desrosier and Heiligman *(3)*.

Furthermore, the spores which are heated and then stored at 2°-4°C. for a period of time, do not spoil tomato juice. These same spores, will,

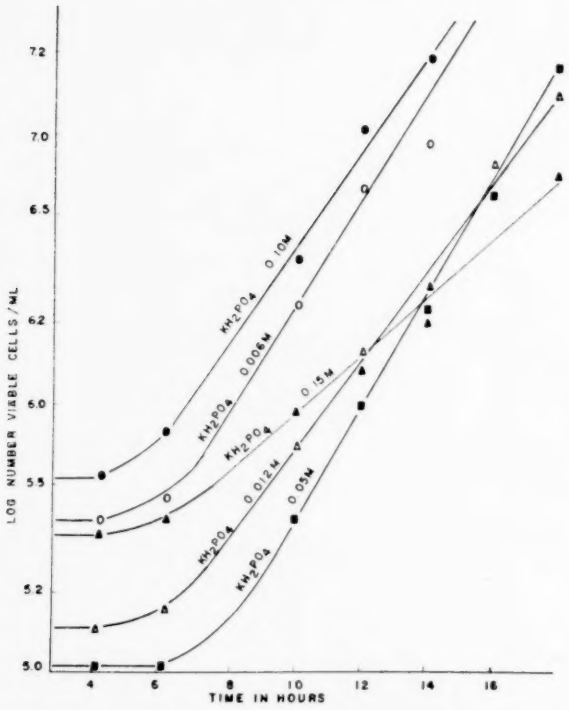

Figure 2. Effects of potassium phosphate concentration on the growth curve of B. thermoacidurans F.S. 787 using vegetative cells for inoculum.

however, spoil tomato juice if the pH of the juice is slightly increased or if potassium phosphate is added and the juice adjusted to its original pH. This indicates that the storage and/or the heat treatment had affected the phosphate metabolism of the spores. Phosphate has been shown by others, Knaysi *(5)* and Powell *(9)*, to be stimulatory to the spore germinating processes. The results obtained (Figures 1, 2) showed that for *B. coagulans* the concentration of potassium phosphate affected the lag-phase of growth when spores were used as inoculum but had little effect when vegetative cells were used. It appears (Figures 3 and 4) that it is the phosphate ion which is critical in the spore germinating process since the other ions tested had little effect.

The phosphorus content of tomato juice is relatively low (17 mg./100 g., Kramer, *6)*. These figures indicate that if all the phosphorus existed as phosphate, the phosphate concentration would be only about 0.005 M. These studies show that, under laboratory conditions, spore germination is slow or negligible at phosphate concentrations up to 0.006 M. Thus, if the available phosphate of a tomato product is low, germination likely would not occur.

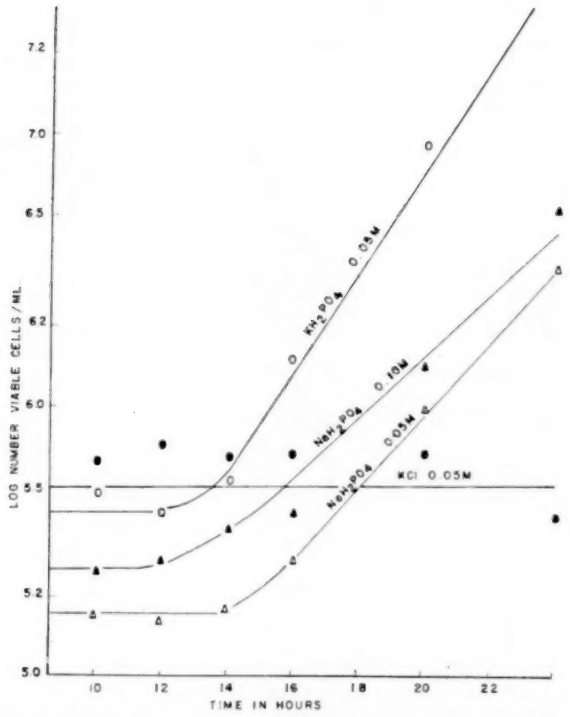

Figure 3. Effects of potassium, sodium, phosphate and chloride on the growth curve of B. thermoacidurans F.S. 787 using spores for inoculum.

The question arises as to the role of phosphate in spore germination and what the effects of phosphate fertilization of growing tomato plants would be on the preparation of commercial tomato products. MacGillivray *(7)* has shown that phosphate fertilization, in normal garden soils, has very little effect on the phosphate concentration of the pulp. He did find, however, that the pulp of tomatoes grown in nutrient solutions high in phosphate had about twice the phosphate concentration of the pulp of the tomatoes grown in phosphate deficient solutions. Further experimentations are needed to determine the role of phosphate in spore germination and the effect of phosphate fertilization on the phosphate content of field grown tomatoes.

SUMMARY

In view of the important bearing that environmental factors have on food spoilage organisms, attempts were made to determine the effect of environment on the cultural characteristics of *B. coagulans* and its germination and growth in tomato juice.

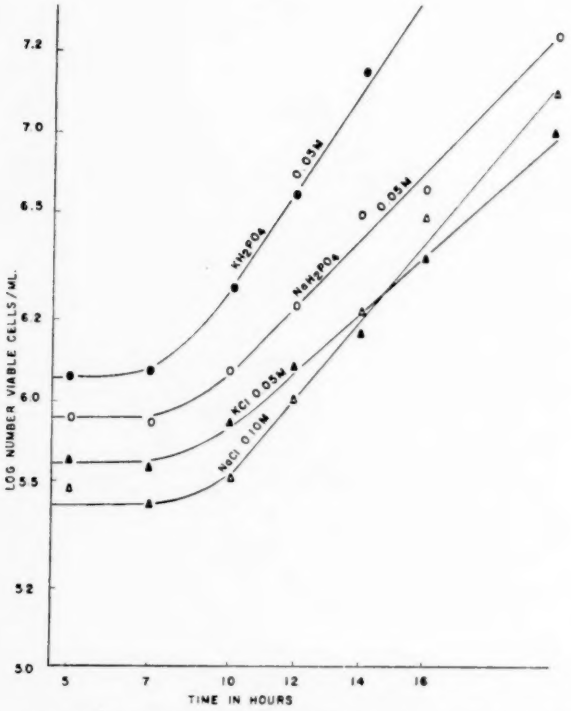

Figure 4. Effects of potassium, sodium, phosphate and chloride ions on the growth curve of B thermoacidurans F.S. 787 using vegetative cells for inoculum.

The results obtained indicate that spores of *B. coagulans* are capable of germinating and growing in tomato juice if they are inoculated immediately after a heat treatment (heat activated spores), or if the pH of the juice is increased.

Spores of *B. coagulans* if heated and stored at 2°-4°C. for a period of time would not spoil tomato juice. However, a slight increase in pH or the addition of potassium phosphate (juice buffered to its original pH) enabled the spores to cause spoilage.

Phosphate ions appear critical in the spore germination process. Further studies are indicated on the role of phosphate in the spore germination process and the effect of phosphate fertilization on the phosphate content of field grown tomatoes.

LITERATURE CITED

1. Becker, M. E., and Pederson, C. S. The physiological characters of *Bacillus coagulans (Bacillus thermoacidurans)*. *J. Bact.*, 59, 717-725 (1950).
2. Breed, R. S., Murray, E. G. D., and Hitchins, A. P. *Bergey's Manual of Determinative Bacteriology*. 6th ed., 1948. Williams and Wilkins Co., Baltimore, Md.

3. DESROSIER, N. W., AND HEILIGMAN, F. Heat activation of bacterial spores. In press, this journal (1955).
4. GORDON, R. E., AND SMITH, N. R. Aerobic spore forming bacteria capable of growth at high temperatures. *J. Bact.*, **58**, 327-341 (1949).
5. KNAYSI, G. Preliminary observations of germination of spores of *Bacillus mycoides* in a nitrogen free medium and certain properties of the transparent cell. *J. Bact.*, **55**, 753-757 (1948).
6. KRAMER, A. Nutritive value of canned foods. V. Proximate and mineral composition. *Food Research*, **11**, 391-398 (1946).
7. MACGILLIVRAY, H. H. Effects of phosphorus on the tomato plant. *J. Agr. Res.*, **34**, 97-129 (1927).
8. PEDERSON, C. S., AND BECKER, M. E. Flat-sour spoilage of tomato juice. New York State Agr. Sta. *Tech. Bull.* 287 (1949).
9. POWELL, J. F. The sporulation and germination of a strain of *Bacillus megatherium*. *J. Gen. Microbiol.*, **5**, 993-1000 (1951).
10. RICE, A. C., AND PEDERSON, C. S. Factors influencing growth of *Bacillus coagulans* in canned tomato juice. I. Size of inoculum and oxygen concentration. *Food Research*, **19**, 124-133 (1954).

HEAT-ACTIVATION OF BACTERIAL SPORES[a, b]

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Sub-lethal heating of certain bacterial spores (Curran and Evans, *4)* and certain fungal spores (Goddard, *8;* Robbins, Kavanaugh, and Kavanaugh, *15)* is reported to accelerate their rate of germination. Curran and Evans *(5)* demonstrated a rapid change in the apparent viability of spores which had been given a sub-lethal heat treatment and stored under conditions unfavorable for germination. From these results they postulated that the sub-lethal heat treatment activated the spores to an intense metabolic activity. Curran and Evans *(6)* also observed, in a given spore suspension, that a greater number of spores germinated if the suspension was given a mild heat treatment before plating.

TABLE 1

Thermal activation and inactivation, and the loss of thermal activation upon storage of spore suspensions of B. thermoacidurans F.S. 787

Time (min)	Temperature of activation: 95°C. Plate count before storage	% Recovery	Plate count after storage	85°C. Plate count before storage	% Recovery	Plate count after storage
0	3.9[1]	.....	3.7	4.1	.....	3.8
2	5.1	131	3.21	4.7	114	3.2
5	3.6	92	2.80	5.2	127	3.7
10	2.71	69	1.46	4.4	107	1.61
20	1.52	39	0.56	2.18	53	0.94
40	0.05	2	0.07	1.20	29	0.222
60	0.00	0	0.00	0.84	20	0.006

Time (min)	Temperature of activation: 75°C. Plate count before storage	% Recovery	Plate count after storage	65°C. Plate count before storage	% Recovery	Plate count after storage
0	3.6	.....	3.5	3.9	.....	4.0
2	3.7	104	3.2	4.8	123	3.9
5	3.4	95	3.3	4.6	118	3.7
10	3.9	108	2.38	4.8	123	3.2
20	4.2	117	1.70	5.9	151	3.2
40	2.34	64	0.72	7.8	200	2.68
60	1.22	34	0.44	5.6	143	2.14

[1] Numbers represent plate counts × 10^3/ml. plated in TA agar, incubation at 50°C. for 48 hours.

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This activation by heat, which can be measured by the degree of germination in a controlled environment, may be due to the stimulation of some essential system and/or materials associated with the spore germinating processes; or, it may be due to the destruction of some factor which is toxic to the spores. If this activation is due to the destruction of a toxic factor, the activation should persist after storage and a time-temperature relationship for maximum activation would not likely exist. However, if the activation is due to the stimulation of some intrinsic factor, a time-temperature relationship for maximum activation should exist. The spores should also lose this stimulation when stored, and they could be reactivated with subsequent heat treatments.

In view of these considerations, and their implications with regard to the processing of foods by heat, a series of experiments was performed to study this phase of spore biology.

PROCEDURE

Preparation of spore suspensions. For these studies, representatives of 3 groups of food spoilage bacteria were chosen, *Bacillus thermoacidurans* F.S. 787 (described in the first of this set of studies as closely resembling *B. coagulans*), *Bacillus globigii*, and Putrefactive Anaerobe No. 3679.[4] Vegetative-cell-free spore suspensions were prepared

TABLE 2

Thermal activation and inactivation, and the loss of thermal activation, upon storage of spores of B. globigii

Time (min)	Temperature of activation: 85°C. Plate count before storage	% Recovery	Plate count after storage	75°C. Plate count before storage	% Recovery	Plate count after storage
0	3.6[1]	....	3.6	3.1	.....	2.85
2	2.72	76	2.68	4.6	148	2.92
5	2.00	56	1.85	4.3	138	2.20
10	1.35	38	1.02	4.0	129	1.58
20	0.32	9	0.26	1.98	64	0.85
40	0.19	6	0.025	1.12	36	0.27
60	0.08	2	0.00	0.52	17	0.08

Time (min)	Temperature of activation: 65°C. Plate count before storage	% Recovery	Plate count after storage	55°C. Plate count before storage	% Recovery	Plate count after storage
0	3.4	.....	3.7	3.2	.....	2.27
2	3.2	92	3.4	3.6	112	2.04
5	4.4	128	2.99	3.2	100	2.42
10	5.2	162	2.26	3.8	119	2.20
20	2.8	61	1.60	5.6	175	1.75
40	1.86	55	0.75	4.2	134	1.34
60	1.05	31	0.28	3.3	106	0.90

[1] Numbers represent plate count × 10^3/ml., plated in TGE agar, incubation at 37°C. for 48 hours.

[4] *B. thermoacidurans* F.S. 787 obtained from the Continental Can Co., Chicago, Ill.; *B. globigii*, from the Department of Biology, Purdue University, Lafayette, Indiana; and P.A. 3679, from the National Canners Assoc., Washington, D. C.

by exposing the harvested cells to ultra-sonic radiation (ca 102 watts, 5 minutes) (Heiligman *et al., 9)*. Since the concentration of spores might have an effect on the results, the spore suspensions were counted and diluted to the desired concentration (2×10^8 spores/ml.) These suspensions were kept at 2° to 4°C.

Treatments. The number of spores which responded to a given treatment was counted using the same techniques as previously described (Heiligman *et al., 9)*. Aliquots of the spore suspensions were plated to determine the viable cell counts of the non-heated spore suspensions. Approximately 20 ml. of the spore suspensions, in sterile test tubes, were heated in a boiling water bath so as to quickly raise the temperature of the suspensions to the desired levels. As soon as this temperature was reached, the tubes were transferred to constant temperature water baths at the desired temperatures. Aliquots for counting were taken periodically for viable cell determinations, portions of each of these aliquots were saved and given the same treatments after storage at room temperature for 12 days.

RESULTS

The response of the spores to the treatments (Tables 1, 2, and 3) show that some spores did not germinate, under the conditions of the experiments, unless they were activated, as higher counts were obtained after the suspensions had been heated. The degree of the response was dependent upon both the temperature of exposure and the time of exposure. It also appears that when a spore suspension is activated to a

TABLE 3

Thermal activation and inactivation, and the loss of thermal activation, upon storage of spores of P.A. 3679

Time (min)	Temperature of activation: 95°C. Plate count before storage	% Recovery	Plate count after storage	85°C. Plate count before storage	% Recovery	Plate count after storage
0	1.82[1]	.....	1.75	1.64	.....	1.80
2	2.21	122	1.71	1.70	103	1.64
5	2.46	133	1.42	2.22	136	1.55
10	2.18	120	1.22	2.80	170	1.38
15	1.74	96	.....	2.68	162	.....
20	1.81	100	0.98	1.04	63	1.24
30	1.33	73	.....	1.98	119	.....
40	1.36	75	0.54	1.80	107	0.80
60	0.68	37	0.30	1.11	68	0.54

Time (min)	Temperature of activation: 75°C. Plate count before storage	% Recovery	Plate count after storage	65°C. Plate count before storage	% Recovery	Plate count after storage
0	1.70	.....	1.65	1.78	.....	1.60
2	2.10	123	1.68	1.70	96	1.72
5	2.49	147	1.50	2.11	118	1.58
10	2.60	153	1.24	2.26	127	1.50
15	3.14	184	.....	2.72	153	.....
20	3.08	181	1.10	2.86	161	1.42
30	2.69	158	.....	3.6	201	.....
40	2.63	155	0.78	3.2	180	1.48
60	1.82	106	0.58	3.3	185	1.24

[1] Numbers represent plate count × 10^8/ml., plated in Brewer's agar, incubation at 37°C. for 48 hours.

maximum response, regardless of the temperature and time, additional heating acts as a lethal agent. Thermal Activation and Rate of Destruction Curves for *B. thermoacidurans* F.S. 787 at the different temperatures, are shown in Figure 1.

The results of viable cell counts of the various heated spore suspensions after they had been stored at room temperature for 12 days are shown in Tables 1, 2, and 3. In all cases the counts were lower than the counts immediately after heating, and the non-heated controls. These relationships for *B. thermoacidurans* F.S. 787 are shown graphically in Figure 2.

The stimulation of a second heat treatment on the spores, which had been previously heated and stored, was similar to the original heat treatments but not as pronounced. The results of the second treatment of the suspension of *B. thermoacidurans* F.S. 787 which had originally been heated for 5 minutes at 85°C. are shown in Table 4 and graphically in Figure 3.

It might be pointed out that the germination of spores, while in storage, may be assumed to be negligible because the viable counts of the suspensions were lower after storage than immediately after heating.

DISCUSSION AND CONCLUSIONS

The stimulatory action of lethal chemical agents in dilute concentrations on biological entities has been demonstrated numerous times. That

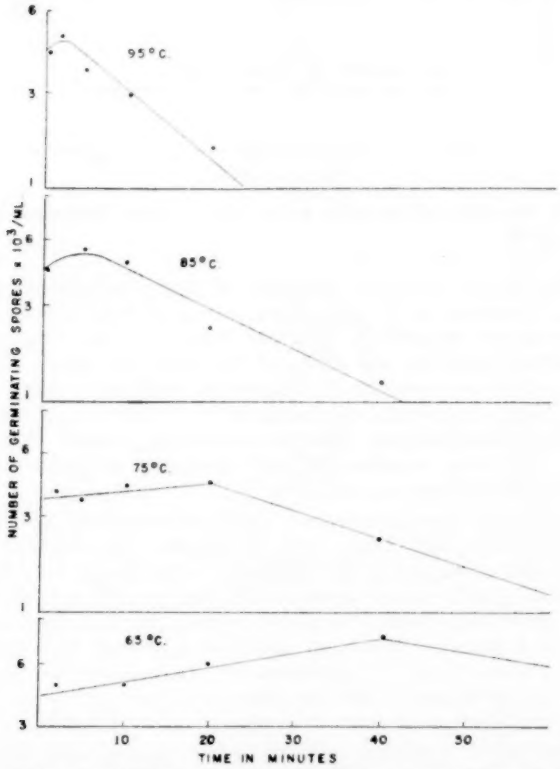

Figure 1. Thermal activation and rate of destruction curves of spore suspensions of B. thermoacidurans F.S. 787 at different temperatures.

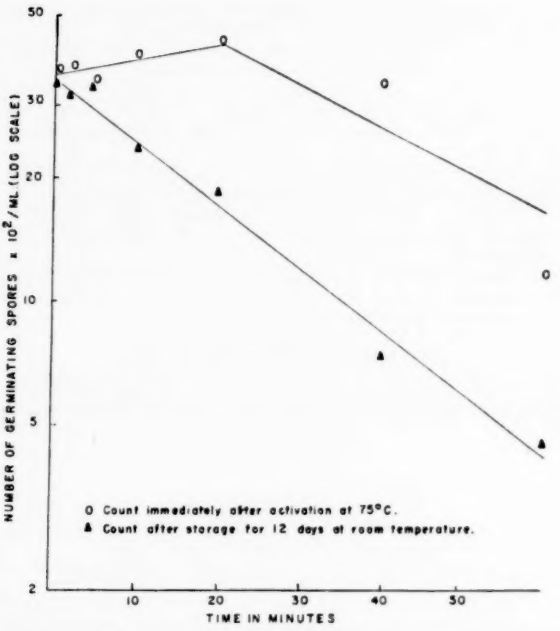

Figure 2. The effects of storage on a heat activated spore suspension of B. thermoacidurans F.S. 787.

there should be an analogous operation of this principle for heat seems reasonable. Evidence of the stimulatory action of mild heat on bacterial-spore enzymes are reported by Cook *(2)*, Tarr *(17)*, and Crook *(3)*. Tarr *(17)* concluded that the real effect of the heat was associated with the reduction in time required for the enzymatic reactions to reach a constant velocity. These views were substantiated by Crook *(3)*. Evidence from the present studies indicates that the accelerated activity of spores by a mild heat shock may be associated with the energy of activation of materials associated with germination.

The results of these experiments are in close agreement with similar studies by Curran and Evans *(6)*. It appears that without sub-lethal heating a substantial portion of potentially viable spores do not germinate when placed in a favorable environment. Apparently not all spores are alike in a given suspension, because their response, as determined by germination in a given environment, differs even though they were formed and treated under identical circumstances. These views are well substantiated by the numerous studies on spore resistance which show that not all spores are killed at the same time when given identical heat treatments.

The problem of dormancy or delayed germination must be considered in any studies of this nature. In the present studies, emphasis was placed on the effects of sub-lethal heat on the germinating processes. Since the

recovery environment was kept constant, those factors which could be attributed to the environment were not considered.

The results of these studies indicate there is a time-temperature relationship for obtaining a maximum stimulation of germination activity by heat-activation. For *B. thermoacidurans* F.S. 787, the maximum recovery at 95°C. occurred when the suspension was treated for only 2 minutes; at 85°C., in 5 minutes; at 75°C. in 20 minutes; and at 65°C. in 40 minutes. Although similar results were obtained for the other strains, the time and temperature relations for maximum stimulation differed (Tables 2 and 3).

The logarithm of the time at which the highest per cent of recovery for a given treatment occurred was plotted against temperature (Figure 4). The logarithmic nature of the curve indicates a first order reaction. This type of time-temperature relationship has its counterpart in Thermal Death Time Curves (Biglow, *1)*.

The rates of reaction for maximum stimulation of the various strains differed. This difference may be related to the heat resistance of the spores. The relative heat resistance of the spores of the different strains is as

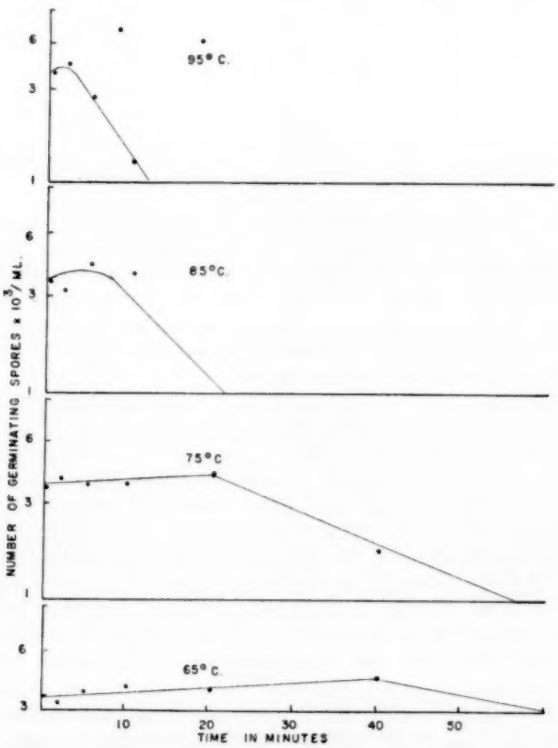

Figure 3. Thermal activation and rate of destruction curves of a spore suspension of B. thermoacidurans F.S. 787 which had been previously heated with sub-lethal heat.

TABLE 4

Thermal activation and inactivation of spore suspensions of B. thermoacidurans F.S. 787 which had previously been activated with heat[1]

Time (min)	Temperature 95°C. Plate count	95°C. % Recovery	85°C. Plate count	85°C. % Recovery
0	3.7[2]	.....	3.7	.....
2	3.9	105	3.5	95
5	2.7	73	4.2	113
10	1.32	36	3.9	105
20	.57	14	0.84	24
40	0.00	00	.131	3
60	0.00	00	0.000	0

Time (min)	Temperature 75°C. Plate count	75°C. % Recovery	65°C. Plate count	65°C. % Recovery
0	3.7	.....	3.7	.....
2	4.0	108	3.5	95
5	3.9	105	3.7	100
10	3.9	105	4.1	111
20	4.2	113	4.0	108
40	1.88	51	4.6	124
60	0.68	18	3.2	87

[1] Spore suspension activated for 5 minutes at 85°C. and stored for 12 days at room temperature.
[2] Numbers represent plate count $\times 10^8$/ml. plated in TA agar, incubation at 50°C. for 48 hours.

follows: P.A. 3679 > *B. thermoacidurans* F.S. 787 > *B. globigii*. A comparison of the rates of activation in the Thermal Activation Time Curves (Figure 4) indicates that the rates of activation are in the reverse order, i.e., *B. globigii* > *B. thermoacidurans*, F.S. 787 > P.A. 3679. From these observations it appears that the factors which are associated with the process of thermal activation of spores may be related to the factors associated with the process of thermal death.

The observation that spores which have been stimulated by heat lose this stimulation when stored suggests that some material, either in or associated with the spores, requires some energy of activation in order to enter into the spore germinating process. This hypothesis can be further substantiated by the fact that these same spores will respond to subsequent heat-activation treatments (Table 3, Figure 3). If this stimulation were the result of the inactivation of toxic factors, as suggested by Mefferd and Campbell *(13)*, the action of the heat should persist and spores should not lose the effect upon storage. It is possible, however, that some anti-germination factor, present in or associated with the spores, is destroyed by the heat, and that this factor can be synthesized by the spores when stored.

A few biologically active materials have been shown to exist in the spore. Knaysi, Baker, and Hillier *(10)* reported that spores contain a nitrogenous material which had the physiological and staining properties of ribonucleic acid. Amino acids have been shown to be present in spores (Davis and Williams, *7*; Lawrence and Halvorson, *11)*. Dipicolinic acid

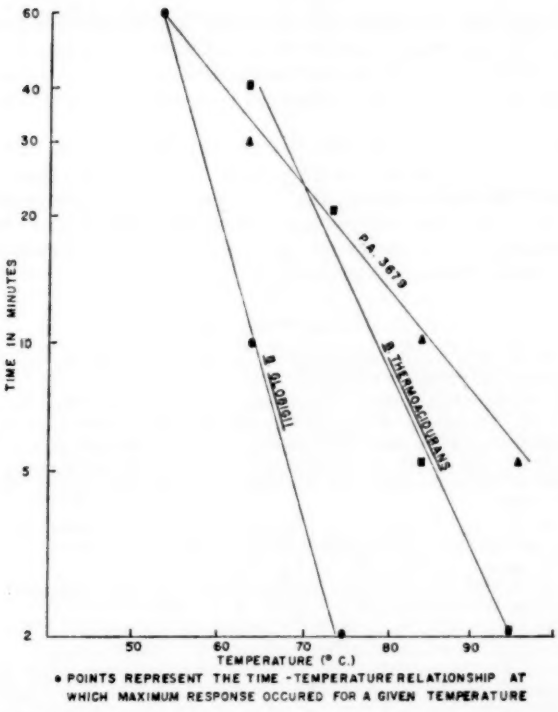

Figure 4. Thermal activation time curves of spore suspensions of B. thermoacidurans, B. globigii and P.A. 3679.*

has been shown to be excreted by spores as they germinate (Powell, *14*). Enzymes have been shown by several workers to exist in spores (Stewart and Halvorson, *16;* Levinson and Sevag, *12*).

It is entirely conceivable that some such materials may be related to the heat-activation of spores. Most spores germinate without heating. These may contain ample concentrations of these biologically active materials and enough of the molecules are in a reactive state to initiate the germination reaction without any energy of activation. On the other hand, spores which contain relatively low concentrations of these materials may be activated with the energy supplied by preheating. It is also possible that heat activation may increase the rate of various reactions that precede and/or are necessary for the germination of the spore.

SUMMARY

The sub-lethal heating of certain bacterial spores is reported to accelerate their rate of germination. Attempts were made to determine the effect of preheating upon the germination of *Bacillus thermoacidurans* F.S. 787, *Bacillus globigii*, and P.A. 3679 spores.

It was found that for each strain of spores there was a time-temperature relationship for obtaining a maximum stimulation of germination activity by heat activation. Furthermore, heat activated spore suspensions lost their stimulation upon storage and could be reactivated with a subsequent heat treatment.

It appears that factors which effect heat activation are related to the factors which are associated with the process of the thermal death of spores.

The results obtained suggest that for some of the spores which do not germinate in a favorable environment, sub-lethal heating may supply the energy of activation or increase the rate of various reactions which precede and/or are necessary for the germination process.

LITERATURE CITED

1. Biglow, W. D. The logarithmic nature of thermal death time curves. *J. Infectious Diseases*, **29**, 528-536 (1921).
2. Cook, R. P. Bacterial Spores. *Biol. Revs.*, Cambridge Phil. Soc., **7**, 1-23 (1932).
3. Crook, P. G. The effects of heat and glucose on endospore respiration utilizing a modified Scholander microrespirometer. *J. Bact.*, **63**, 193-198 (1952).
4. Curran, H. R., and Evans, F. R. Heat activation inducing germination in bacterial spores of thermoltolerant and thermophilic aerobic bacteria. *J. Bact.*, **45**, 335-346 (1945).
5. Curran, H. R., and Evans, F. R. The viability of heat-activated in nutrient and non-nutrient substrates as influenced by prestorage and poststorage heating and other factors. *J. Bact.*, **53**, 103-113 (1947).
6. Curran, H. R., and Evans, F. R. Heat activatable spores in evaporated milk. *Food Inds.*, **20**, 370-371 (1948).
7. Davis, F. L., and Williams, O. B. Chromatographic analysis of the amino acid composition of bacterial spores. *J. Bact.*, **54**, 766-767 (1952).
8. Goddard, D. R. The reversible heat activation inducing germination and increased respiration in the ascospores of *Neurospora* tetrasperma. *J. Gen. Physiol.*, **19**, 45-60 (1935).
9. Heiligman, F., Desrosier, N. W., and Broumand, H. Preparation of vegetative-cell-free spore suspensions without the use of heat. (This journal). (1955).
10. Knaysi, G., Baker, R. F., and Hillier, J. A study, with the high-voltage electron microscope, of the endospore and the life cycle of *Bacillus mycoides*. *J. Bact.*, **53**, 525-537 (1947).
11. Lawrence, H. L., and Halvorson, H. O. Studies on spores of aerobic bacteria. III. The D-amino acid content of spores and vegetative cells of *Bacillus terminalis*. *J. Bact.*, **67**, 585-588 (1954).
12. Levinson, H. S., and Sevag, M. G. Stimulation of germination and respiration of spores of *Bacillus subtilis* by manganese and univalent ions. *J. Gen. Physiol.*, **36**, 617-629 (1953).
13. Mefferd, R. B., and Campbell, L. L. The activation of thermophilic spores by furfural. *J. Bact.*, **62**, 130-132 (1951).
14. Powell, J. F. Isolation of dipicolonic acid (pyridine-2, 6-dicarboxylic acid) from spores of *Bacillus megaterium*. *Biochem J.*, **54**, 210-211 (1953).
15. Robbins, W. T., Kavanaugh, V. W., and Kavanaugh, F. Growth substances and dormancy of spores of Phycomyces. *Bot. Gaz.*, **104**, 224-242 (1942).
16. Stewart, R. T., and Halvorson, H. O. Studies on spores of aerobic bacilli. I. The occurrence of alanine racemase. *J. Bact.*, **65**, 160-166 (1953).
17. Tarr, H. L. A. Some observations on the respiratory catalysts present in the spores and vegetative cells of certain aerobic bacteria. *Biochem. J.*, **27**, 136-145 (1933).
18. Topley, W. W. C., and Wilson, G. S. *The Principles of Bacteriology and Immunology*. 2nd ed., W. Wood and Co., Baltimore, Md.

SPORE GERMINATION. I. ACTIVATORS[a,b]

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(Manuscript received July 15, 1955)

The recent physiological studies of Hills *(3)* and Powell *(9)* have shown that biologically active materials are essential for spore germination. Evidence that the spore needs only a source of energy, such as glucose, has been presented by Knaysi *(7)*. The views that only exogenous energy is stimulatory to the spore germinating processes are not fully substantiated by the work of Hills *(4)* and Stewart and Halvorson *(12)*. These workers have presented good evidence that L-alanine is a factor which will initiate spore germination. Undoubtedly species specificity will enter into studies of this nature, as L-alanine has not been shown to function in all of the cases that have been studied.

The following studies deal with the effects of naturally occurring materials, particularly those which can be utilized as a carbon source for energy, on the germinating processes.

MATERIALS AND METHODS

Spore suspensions. Spores of a strain of *Bacillus thermoacidurans*, F.S. 787, closely in agreement with the description of *B. coagulans* (Hammer), were used primarily in these studies; however, spores of *Bacillus globigii* and Putrefactive Anaerobe No. 3679[d] were included in some of the experiments in order to determine if the results were characteristic of one or more species. The cell suspensions were prepared as described in a previous report of Heiligman *et al. (2)*. Ultra-sonic radiation (approximately 102 watts for 5 minutes) was used in preparing the vegetative-cell-free spore suspensions. The concentration of the spores was kept constant (approximately 20,000 per ml.) so as to eliminate any effects due to this factor.

Criterion of germination. As suggested by Wynne and Foster *(13, 14)* spores which had lost their resistance to heat were considered as having germinated. Quantitative results were based on the difference, calculated in percent, in the number of viable cells in an aliquot after a germination incubation treatment as compared to the viable cell count of a similar aliquot after it had been heated at 100°C. for one minute. The former count represents the total count of all the viable cells, both those which had and those which had not lost their resistance to heat; the latter represents a count of the residual viable spores. The difference between these counts, then, would be the number of spores which had lost their resistance to heat as a result of the incubation germination treatment.

Treatment of spores. The spores were added aseptically to sterile solutions of the materials. These suspensions were given a germination incubation at the desired temperature and aliquots for counting were taken periodically. The germination incubation temperature, except for some preliminary studies on *B. thermoacidurans*, was 50°C. for this organism, and 37°C. for *B. globigii* and P.A. 3679. Since P.A. 3679 is an anaerobe

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[b] Presented at the 15th Annual Meeting, IFT, Columbus, Ohio, June 14, 1955.

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[d] *B. thermoacidurans* F.S. 787 obtained from Continental Can Co., Chicago, Ill.; *B. globigii* from the Department of Biology, Purdue University, Lafayette, Ind.; P.A. 3679 from National Canners Association, Washington, D. C.

TABLE 1

The percent germination of spores of B. thermoacidurans in the constituents of TA broth[1]

Substrate	Incubation time (hrs) at 67°C. 7	13	24	32	43	58	74	140
Phosphate buffer	6[2]	9	21	10	24	45	62	66
Glucose	11	43	77	89	94	99	99	99
Proteose peptone	9	27	43	64	84	90	99	99
Yeast extract	9	15	59	77	87	91	99	99
Glucose and yeast extract	6	47	76	90	96	99	99	99
Glucose and proteose peptone	12	45	81	92	95	99	99	99
Yeast extract and proteose peptone	13	29	45	66	80	87	99	99
Glucose, yeast extract, and proteose peptone	15	51	78	99	99	99	99	99

[1] Phosphate buffer (0.025M, pH 6.0); glucose (0.5%); proteose peptone (0.5%); yeast extract (0.5%).
[2] Percent germination.

and atmospheric oxygen is extremely toxic to the vegetative cells (Wynne, Mehl, and Schmeidling, *16)*, spores of this organism were incubated in an atmosphere of nitrogen. The plating techniques, for determining the results of the various treatments, were the same as in a previous report (Heiligman *et al., 2)*.

RESULTS

The following materials were used as substrate for spore germination.

Constituents of Thermoacidurans Broth: Solutions of the individual constituents of TA broth,* singly and in all possible combinations, were inoculated with spores of *B. thermoacidurans* and given a germination incubation treatment at 67°C. The results are shown in Table 1.

Sugars and Related Carbon Compounds: Table 2 shows the results of a series of similar experiments, using sugars and energy yielding carbon compounds in dilute solutions.

Glucose and Intermediate Metabolites of Carbohydrate Metabolism: Spores of the three test species were tested for their ability to germinate in glucose and some of the intermediate acids which occur in the metabolism of glucose. These materials were used in the same concentration as glucose. The activity was tested at pH 4.7, as well as pH 7.0 (Tables 3 and 4).

Fatty Acids: Several fatty acids, including acetic, propionic, butyric, caproic, caprylic, myristic, oleic, palmitic, and stearic acids, were tested for their ability to serve as substrates for the germination of spores of *B. thermoacidurans* F.S. 787. The soluble acids were used in 0.3 M. (pH 7.0) concentrations; the insoluble acids were used in super-saturated solutions in phosphate buffer (pH 7.0). The results are shown in Table 5.

Nitrogenous Materials: Several nitrogen containing materials, both organic and inorganic, were tested for their ability to serve as substrates for the germination of spores of *B. thermoacidurans* F.S. 787. These included potassium nitrate, ammonium nitrate, ammonium chloride, L-, DL-, and D-alanine, L-aspartic acid, L-glutamic acid,

* Glucose (0.5%), proteose peptone (0.5%), yeast extract (0.5%), in phosphate buffer (0.025 M, pH 6.0).

TABLE 2

The percent germination of spores of B. thermoacidurans in sugars and related carbon compounds[1]

Substrate	Incubation time (hrs) at 67°C. 6	24	48
Phosphate buffer (0.025M)	3[2]	15	28
Phosphate buffer (0.00025M)	4	12	25
Glucose (0.03M)	55	97	99
Glucose (0.03M, pH 4.7)	57	98	99
Glucose (0.03M in H_2O)	13	27	36
Fructose (0.03M)	52	93	99
Galactose (0.03M)	33	90	97
Arabinose (0.03M)	5	17	31
Xylose (0.03M)	7	20	30
Lactose (0.03M)	46	79	94
Maltose (0.03M)	54	95	99
Sucrose (0.03M)	56	97	99
Starch (0.05%)	47	77	92
Glycerol (0.06M)	5	18	36
Mannitol (0.03M)	13	15	18
Sorbitol (0.03M)	43	72	88
Fumaric acid (0.04M)	46	71	88
Malic acid (0.04M)	44	68	85
Succinic acid (0.04M)	38	59	75
Citric acid (0.03M)	44	68	86
Sodium pyruvate (0.06M, pH 7.0)	19	28	40
Sodium pyruvate (0.06M, pH 4.7)	53	80	92

[1] In phosphate buffer (0.025M, pH 7.0 unless specified).
[2] Percent germination.

TABLE 3

The percent germination of spores of B. thermoacidurans in glucose and intermediate acids[1] of the anaerobic and Kreb's cycles

Substrate (pH 7.0)	Incubation time (hrs) at 50°C. 2	5	10	24
Glucose (0.3M)	11[2]	37	58	99
Glyceric acid (0.6M)	4	9	26	70
Sodium pyruvate (0.6M)	6	7	10	29
Citric acid (0.3M)	7	12	29	56
Succinic acid (0.4M)	4	14	26	50
Fumaric acid (0.4M)	7	9	21	42
Malic acid (0.4M)	4	11	19	39
Phosphate buffer	2	8	15	21
Substrate (pH 4.7)	Incubation time (hrs) at 50°C. 2	5	10	24
Glucose (0.3M)	8	22	44	95
Glyceric acid (0.6M)	6	11	23	80
Sodium pyruvate (0.6M)	9	14	42	93
Citric acid (0.3M)	8	13	25	81
Succinic acid (0.4M)	3	12	27	83
Fumaric acid (0.4M)	7	16	29	86
Malic acid (0.4M)	4	15	30	87
Phosphate buffer	3	4	7	17

[1] In phosphate buffer (0.025M).
[2] Percent germination.

TABLE 4

The percent germination of spores of B. globigii and P.A. 3679 in glucose and intermediate acids[1] of the anaerobic and Kreb's cycles

B. globigii							
Substrate (pH 7.0)	Incubation time (hrs) at 37°C. 5	10	24	Substrate (pH 4.7)	Incubation time (hrs) at 37°C. 5	10	24
Glucose	62[2]	85	99	Glucose	31	78	92
Sodium pyruvate	11	20	31	Sodium pyruvate	21	58	79
Citric acid	31	49	68	Citric acid	26	57	82
Malic acid	28	43	64	Malic acid	21	54	75
Phosphate buffer	5	10	21	Phosphate buffer	4	9	16
P.A. 3679							
Glucose	32	46	76	Glucose	26	37	69
Sodium pyruvate	4	20	30	Sodium pyruvate	20	35	57
Citric acid	6	40	59	Citric acid	17	29	51
Malic acid	4	18	54	Malic acid	18	32	54
Phosphate buffer	2	9	13	Phosphate buffer	2	3	8

[1] In phosphate buffer (0.025M); glucose (0.3M), sodium pyruvate (0.6M), citric acid (0.3M), malic acid (0.4M).
[2] Percent germination.

TABLE 5

The percent germination of spores of B. thermoacidurans in solutions of fatty acids[1]

Substrate	Incubation time (hrs) at 50°C. 6	10	24	Substrate	Incubation time (hrs) at 50°C. 6	10	24
Glucose	22[2]	57	99	Acetic acid	12	20	28
Propionic acid	8	13	27	Butyric acid	10	17	24
Caproic acid	5	10	21	Caprylic acid	4	8	16
Myristic acid	6	9	22	Oleic acid	7	11	17
Palmitic acid	4	8	18	Stearic acid	5	12	20

[1] In phosphate buffer (0.025M, pH 7.0, acetic, propionic and butyric acids (0.3M), caproic, caprylic, myristic, oleic, palmitic, and stearic acids (super-saturated solution).
[2] Percent germination.

DL-tyrosine, and adenosine. In some of the treatments, glucose was incorporated with the nitrogenous material. All compounds were in solution in phosphate buffer (0.025M, pH 7.0) (Table 6).

DISCUSSION

Spore forming bacteria, especially thermophiles, are usually very exacting in their nutritional requirements for growth *(1)*. Yet these studies show that the spores can germinate, as defined by loss of heat resistance, in environments unfavorable for the growth of the vegetative cells. The observation that germination will proceed, if given only a source of energy, suggests that the spore is an entity complete within itself. These studies,

TABLE 6

The percent germination of spores of B. thermoacidurans in nitrogen containing solutions[1]

Substrate	Incubation time (hrs) at 50°C. 5	10	24	Substrate	Incubation time (hrs) at 50°C. 5	10	24
Glucose	34[2]	56	99	Phosphate buffer	7	14	20
Potassium nitrate	5	9	23	Ammonium nitrate	9	13	22
Ammonium chloride	6	12	25	Adenosine	8	16	24
L-alanine	20	34	52	L-glutamic acid	7	16	28
DL-alanine	6	11	26	L-aspartic acid	9	15	26
D-alanine	5	9	22	DL-tyrosine	9	14	22
L-alanine, glucose, and adenosine	36	71	99	DL-alanine, glucose, and adenosine	8	15	23

[1] In phosphate buffer (0.025M, pH 7.0); glucose (0.3M), KNO_3 (0.3M), NH_4NO_3 (0.3M), NH_4Cl (0.3M), D, DL, and L-alanine (0.5M), L-aspartic acid (0.5M), L-glutamic acid (0.5M), DL-tyrosine (0.5M), Adenosine (1×10^{-6}M).
[2] Percent germination.

and those of Knaysi *(5)* and Wynne, Mehl, and Schmeidling *(16)* indicate that with the exception of available energy, the spores have all the essential materials needed to initiate the process of germination.

The results indicated in Table 1 show that spores can germinate in a substrate containing only carbohydrates, or closely related compounds, and phosphate. This indicates that spores contain the nitrogenous materials, essential for initiating germination. These findings are in agreement with those of Knaysi *(6)* and Knaysi, Hillier, and Baker *(8)* as these workers found that spores contain relatively large concentrations of nitrogenous materials.

The observation that spores can utilize, for germination, only the sources of carbon that the vegetative cell can utilize for growth suggests that the metabolism of spores is closely associated with the metabolism of the vegetative cell (Table 2). A similar observation has been made by Knaysi *(7)* for spores of *Bacillus mycoides.*

It is of interest to note that, although the germination of *B. thermoacidurans* F.S. 787 proceeded at a high rate if the glucose was in solution in phosphate buffer, this sugar, in an aqueous solution, was not capable of serving as a very satisfactory substrate (Table 2). Therefore, it appears that available phosphate plays an important role in spore germination. Similar observations have been made by Knaysi *(5)* and Powell *(9)*.

These results, along with the observations that spores can utilize carbohydrates and similar energy yielding compounds, add evidence to the theory that spores are complete entities and will germinate in a favorable environment if given a source of energy.

The conversion of glucose and the intermediate acids of the anaerobic and Kreb's cycle into available energy for germination (Table 3) presents additional evidence to this theory. The failure to obtain response from some of these acids at pH 7.0 may have been due to their unavailability to the cell because at this high pH, most of the acids exist in the associated form. This effect of pH on the biological activity of many weak acids and

weak bases has often been noted, and reported by Simon and Beevers *(11)*.

The failure of spores to respond to fatty acids has been noted by Wynne and Foster *(15)* and Roth and Halvorson *(10)*. The failure of the spores of *B. thermoacidurans* F.S. 787 to germinate in solutions of fatty acids indicated that these acids cannot be converted into materials which can be utilized by the spore.

The results of the experiments in which the amino acids were used as substrates present interesting speculation (Table 5). L-alanine, and only this amino acid, seemed to have any stimulatory activity on the germinating processes. Stewart and Halvorson *(12)*, after isolating an L-alanine racemase from spores of *B. terminalis*, suggested that this racemase was involved in the germinating process. It is difficult to explain the role of this naturally occurring material in spore germination. It is possible that the spore can convert this amino acid into some material, such as pyruvate, which can be utilized as a source of energy but this has not been shown. The results of this series of experiments are in close agreement with Stewart and Halvorson's report *(12)* that D-alanine was inhibitory to the spore germinating processes.

It was observed that the highest rate of germination occurred when glucose, L-alanine and adenosine were combined in the substrate.

SUMMARY

The effect of the following materials upon the spore germination were studied: constituents of TA broth, sugar and the related carbon compounds, glucose and the intermediate metabolites of carbohydrate metabolism, fatty acids, and organic and inorganic nitrogenous compounds. Spores of *B. thermoacidurans* F.S. 787 were used primarily, and in certain experiments spores of *B. globigii* and P.A. No. 3679 were included in order to determine if the results were characteristic of spores in general.

The results obtained indicated that:

Spores could germinate in a substrate containing only carbohydrate or its related compounds which act as a source of energy.

Available phosphate played an important role in spore germination.

Spores did not respond to fatty acid as a source of energy for germination.

From amino acids tested, only L-alanine had any stimulating activity on the germinating processes.

The highest rate of germination occurred when glucose, L-alanine and adenosine were combined in the substrate.

LITERATURE CITED

1. ALLEN, M. B. The thermophilic aerobic bacteria. *Bact. Revs.*, 17, 125-173 (1953).
2. HEILIGMAN, F., DESROSIER, N. W., AND BROUMAND, H. Preparation of vegetative-cell-free spore suspensions without heat. (In press).
3. HILLS, G. M. Chemical factors in the germination of spore bearing aerobes. The effects of yeast extract on the germination *Bacillus anthracis* and its replacement with adenosine. *Biochem. J.*, **45**, 353-362 (1949).
4. HILLS, G. M. Chemical factors in the germination of spore bearing aerobes. The effects of amino acids on the germination of *Bacillus anthracis*, with some observations on the relation of the optical forms to biological entities. *Biochem. J.*, **45**, 363-370 (1949).

5. KNAYSI, G. A study of some environmental factors which control endospore formation by a strain of *Bacillus mycoides*. *J. Bact.*, **49**, 473-493 (1945).
6. KNAYSI, G. Investigation on the existence and nature of reserve materials in the endospore of *Bacillus mycoides* by an indirect method. *J. Bact.*, **49**, 617-622 (1945).
7. KNAYSI, G. Preliminary observations of germination of spores of *Bacillus mycoides* in a nitrogen free medium and certain properties of the transparent cell. *J. Bact.*, **55**, 753-757 (1948).
8. KNAYSI, G., BAKER, R. F., AND HILLIER, J. A study, with the high voltage electron microscope of the endospore and the life cycle of *Bacillus mycoides*. *J. Bact.*, **53**, 525-537 (1947).
9. POWELL, J. F. The sporulation and germination of a strain of *Bacillus megaterium*. *J. Gen. Microbiol.*, **5**, 993-1000 (1951).
10. ROTH, N. G., AND HALVORSON, H. O. The effects of oxidative rancidity in unsaturated fatty acids on the germination of bacterial spores. *J. Bact.*, **63**, 429-435 (1952).
11. SIMON, W. W., AND BEEVERS, H. The effects of pH on the biological activity of weak acids and bases. I. The most unusual relationship between pH and activity. *New Phytologist*, **51**, 163-190 (1952).
12. STEWART, B. T., AND HALVORSON, H. O. Studies on spores of aerobic bacteria. I. The occurrence of alanine racemase. *J. Bact.*, **65**, 160-166 (1953).
13. WYNNE, E. S., AND FOSTER, J. W. Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of quantitative methods. *J. Bact.*, **55**, 61-68 (1948).
14. WYNNE, E. S., AND FOSTER, J. W. Physiological studies on spore germination with special reference to *Clostridium botulinum*. II. Quantitative aspects of the germination process. *J. Bact.*, **55**, 69-73 (1948).
15. WYNNE, E. S., AND FOSTER, J. W. Physiological studies on spore germination with special reference to *Clostridium botulinum*. IV. Inhibition of germination by unsaturated C-18 fatty acids. *J. Bact.*, **55**, 495-501 (1948).
16. WYNNE, E. S., MEHL, D. A., AND SCHMEIDLING, W. R. Germination of *Clostridium* spores in buffered glucose. *J. Bact.*, **67**, 435-437 (1954).

SPORE GERMINATION. II. INHIBITORS[a, b]

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In a previous paper *(4)*, the effect of certain activators upon spore germination were studied. The effect of some inhibitors of spore germination are reported below.

MATERIAL AND METHODS

Spore suspensions. Spores of *Bacillus thermoacidurans* F.S. 787[d] were used for these studies. The methods of preparation and the treatment of the spores were similar to those described by Heiligman *et al. (4)*.

Criterion of germination. As suggested by Wynne and Foster *(13, 14)*, spores which had lost their resistance to heat were considered to have germinated. Quantitative results were based on the difference, calculated in per cent, in the number of viable cells in an aliquot after a germination incubation treatment as compared to the viable cell count of the same aliquot after it had been heated at 100°C. for one minute. The former count represents the total count of all the viable cells, both those which *had* and those which *had not* lost their resistance to heat; the latter represents a count of the residual viable spores. The difference between these counts, then, would be the number of spores which had lost their resistance to heat as a result of the incubation germination treatment.

RESULTS

Inhibition of the effect of the acids on the Kreb's cycle by malonic acid. The results of a series of experiments, so designed to determine if the stimulatory action of Kreb's acids on spore germination could be inhibited by malonic acid are shown in Table 1. The results of quantitative inhibition studies, using citric and malic acids as substrate and malonic acid in three concentrations (0.03M, 0.003M, and 0.0003M) are shown in Table 2.

TABLE 1

The per cent germination of spores of B. thermoacidurans F.S. 787 in the Kreb's acids[1] as affected by malonic acid

Substrate	Incubation time (hrs) at 50°C. 6	10	24	Substrate	Incubation time (hrs) at 50°C. 6	10	24
Phosphate buffer	7[2]	12	17	Malonic acid	5	9	14
Glucose	54	72	92	Glucose and malonic acid	36	62	83
Citric acid	40	61	79	Citric acid and malonic acid	10	18	25
Succinic acid	44	59	86	Succinic acid and malonic acid	15	24	28
Fumaric acid	36	58	78	Fumaric acid and malonic acid	29	51	72
Malic acid	49	60	81	Malic acid and malonic acid	26	49	74

[1] In phosphate buffer (0.025M, pH 4.7), glucose (0.3M), citric acid (0.3M), succinic, fumaric, and malic acids, (0.4M), malonic acid (3×10^{-2}M).
[2] Per cent germination.

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[a] Journal Paper No. 875. Purdue University Agricultural Experiment Station, Lafayette, Indiana.

[b] Presented at the 15th Annual Meeting, IFT, Columbus, Ohio, June 14, 1955.

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[d] This organism was obtained through the courtesy of Continental Can Company, Chicago, Illinois.

TABLE 2

The per cent germination of spores of B. thermoacidurans F.S. 787 in citric and malic acids[1] as affected by varying concentrations of malonic acid

Concentration of inhibitor	Citric acid incubation time (hrs) at 50°C. 6	10	24	Malic acid incubation time (hrs) at 50°C. 6	10	24
None	41[2]	62	81	45	64	83
3×10^{-2}M	12	17	24	26	49	72
3×10^{-3}M	9	26	43	34	51	80
3×10^{-4}M	44	57	74	32	53	81

Malonic acid (without substrate) 3×10^{-2}M Time (hrs) 6	10	24
2	4	12

[1] In phosphate buffer (0.025M, pH 4.7); citric acid (0.3M), malic acid (0.4M).
[2] Per cent germination.

Inhibition of the effects of glucose and sodium pyruvate by respiratory inhibitors. A group of known respiratory inhibitors, which included 2,4-dinitrophenol, sodium arsenate, sodium azide, sodium arsenite, sodium fluoride, and iodoacetic acid, were tested to determine if the stimulatory action of glucose and sodium pyruvate on the spore germinating processes of *B. thermoacidurans* could be reversed.

The results of quantitative studies, in which 3 concentrations of each inhibitor were used with both glucose and sodium pyruvate as substrates, are shown in Table 3.

DISCUSSION

That the Kreb's acids will stimulate spore germination was reported by Wynne and Foster *(13, 14)*. The observation (Table 1) that malonic acid can inhibit the action of citric and succinic acids, but has little or no effect on the availability of fumaric and malic suggests that succinic dehydrogenase is involved in the germinating process when Kreb's acids are used as the sole source of energy. Quantitative inhibition studies (Table 2) showed that germination, using citric acid as a substrate, can be quantitatively inhibited by malonic acid. When malic acid was used as the substrate, malonic acid had little or no effect. Since the activity of spore germination in either fumaric or malic acids is not strongly inhibited by malonic acid, the oxidation of either malate or fumarate to succinate appeared to produce the requisite energy for the spores to germinate.

The data in Table 3 indicates that the stimulatory action of either glucose or sodium pyruvate can be inhibited by materials which inhibit the oxidation of glucose or pyruvate. The pattern of inhibition revealed that the inhibitors act differently when these two substrates are used. This difference would be expected and depends upon the mode of action and the sites of inhibition.

The inhibitor 2,4-dinitrophenol seemed to interfere with the stimulatory action of both glucose and pyruvate at about the same level of inhibition. This material reacts by uncoupling the energy rich phosphate bonds (Loomis and Lipmann, *8;* Judah, *6)*. Although glucose or pyruvate may be broken down, no energy seemed to be available for the germination of the spores. Sodium azide, which has a similar mode of action (Loomis and

TABLE 3

The per cent germination of spores of B. thermoacidurans F.S. 787 in glucose and sodium pyruvate[1] as affected by varying concentrations of respiratory inhibitors

Molar concentration of inhibitor	Glucose incubation time (hrs) at 50°C. 1.5	5	10	24	Sodium pyruvate incubation time (hrs) at 50°C. 1.5	5	10	24
None	11[2]	52	74	93	10	40	65	82
2,4-dinitrophenol								
3×10^{-2}	4	10	16	29	7	9	15	21
3×10^{-3}	8	23	37	53	10	21	31	50
3×10^{-4}	9	40	65	88	8	37	62	78
Sodium azide								
3×10^{-2}	3	19	30	49	4	17	27	46
3×10^{-3}	11	31	52	60	8	27	43	65
3×10^{-4}	8	50	69	90	11	37	64	80
Sodium fluoride								
3×10^{-2}	4	16	24	35	7	24	42	64
3×10^{-3}	7	19	32	46	11	31	46	75
3×10^{-4}	11	41	67	88	8	38	62	80
Iodo-acetic acid								
3×10^{-2}	4	12	18	30	5	8	17	24
3×10^{-3}	6	19	32	48	8	17	25	42
3×10^{-4}	9	32	53	81	12	24	43	76
Sodium arsenate								
3×10^{-3}	2	11	19	34	10	12	19	27
3×10^{-4}	4	20	31	51	8	21	28	45
3×10^{-5}	8	40	63	86	10	27	48	77
Sodium arsenite								
3×10^{-2}	4	10	32	47	7	17	25	26
3×10^{-3}	6	24	40	55	8	14	24	41
3×10^{-4}	9	34	65	87	8	36	64	77

Inhibitors without substrate

Inhibitor (3×10^{-2}M)	Incubation time (hrs) 1.5	5	10	24
2,4-dinitrophenol	0	4	4	9
Sodium azide	5	7	11	14
Sodium fluoride	8	11	12	15
Iodo-acetic acid	8	7	11	14
Sodium arsenate	3	5	8	10
Sodium arsenite	6	7	9	16

[1] In phosphate buffer (0.025M pH 4.7); glucose (0.3M), sodium pyruvate (0.6M).
[2] Per cent germination.

Lipmann, *9)* inhibited germination quantitatively, but seemed to have a lower level of inhibition (Table 3).

A comparison of the inhibition of glucose and pyruvate by sodium fluoride (Table 3) shows that stimulatory action of pyruvate was less affected than that of glucose. Since sodium fluoride is a glycolytic inhibitor (James, *5)*, inhibiting the enzyme enolase, the energy produced by the conversion of glucose to enol-pyruvate was apparently not sufficient to initiate the germination of the spores.

The degree of inhibition of germination produced by iodoacetic acid and sodium arsenate indicated that these two inhibitors act in a similar manner in the metabolism of either glucose or pyruvate by the spore. Although iodoacetic acid has been shown to inhibit sulfhydryl enzymes in low concentration, it is also thought that this inhibitor affects phosphorylation (Beevers and Simons, *2;* Beevers, *1*). Sodium arsenate, although not specific in its action, prevents the transfer of phosphate to adenosine-diphosphate, and, consequently, there is no formation of adenosine-triphosphate (Pillai, *10*). If phosphate metabolism were inhibited, energy for germination would not be available from either glucose or pyruvate.

The degree of inhibition produced by sodium arsenite (Table 3) shows that the metabolism of glucose was less sensitive to this inhibitor than was pyruvate. Peters, Sinclair, and Thompson *(12)* have shown that arsenite totally inhibits the oxidative decarboxylation of pyruvic acid and has but little effect on triophosphate dehydrogenase. The enzyme most vigorously attacked has not yet been identified. Apparently, with the use of this inhibitor, small amounts of energy were produced by the metabolism of the spores from glucose, but little or none from the metabolism of pyruvate.

Powell *(11)* was unable to obtain spore formation by vegetative cells of *B. megatherium* in the presence of cyanide, azide, 2,4-dinitrophenol, iodoacetic acid, oxalate or citrate when these materials were buffered at pH 7.3. Mention was made of the possibility that these materials may not enter the cell at this high pH value, but the author still concluded that spore formation required energy and postulated that this energy may be released when the spore germinates. Evidence that spore formation requires exogenous energy has been presented by Hardwick and Foster *(3)*.

Keilin and Hartree *(7)* have shown that cyanide and azide will completely inhibit the respiration of spores *B. subtilis* and that 8-hydroxyquinoline completely inhibits respiration and germination. From these data they concluded that factors which will inhibit the respiration of spores will also inhibit their germination. They further postulated that spores need energy for germination. The present studies are in close agreement with these observations.

SUMMARY

The influence of respiratory inhibitors on the germination of *B. thermoacidurans* F.S. 787 spores was studied.

Malonic acid was used as an inhibitor in substrates which contained acids of Kreb's cycle. It was observed that malonic acid can inhibit the metabolism of citric and succinic acids but has little or no effect on fumaric and malic acids in yielding energy for spore germination.

Studies with known respiratory inhibitors revealed that the stimulatory action of either glucose or sodium pyruvate on spore germination can be inhibited by materials which inhibit the oxidation of glucose or pyruvate, although the patterns of inhibition differ.

The germination of bacterial spores requires energy. The presence of respiratory inhibitors such as 2,4-dinitrophenol, sodium arsenate, sodium azide, sodium arsenite, sodium fluoride, and iodoacetic acid reduced the availability of certain usual energy yielding metabolic pathways, substantially retarding spore germination.

LITERATURE CITED

1. BEEVERS, H. Effects of iodoacetic acid on zymase fermentation. *Nature*, **165**, 975-976 (1949).
2. BEEVERS, H., AND SIMONS, F. W. Effects of pH on the activity of some respiratory inhibitors. *Nature*, **165**, 408-409 (1949).
3. HARDWICK, W. A., AND FOSTER, J. W. On the nature of sporogenesis. *J. Gen. Physiol.*, **35**, 907-927 (1952).
4. HEILIGMAN, F., DESROSIER, N. W., AND BROUMAND, H. Spore Germination I. Activators. *Food Research*, **21** (This issue (1956).
5. JAMES, W. O. The use of respiratory inhibitors. *Ann. Rev. Plant Physiol.*, **4**, 59-90 (1953).
6. JUDAH, J. D. The action of 2,4-dinitrophenol on oxidative phosphorylation. *Nature*, **163**, 408-409 (1951).
7. KEILIN, D., AND HARTREE, E. F. Comparative studies of spores vegetative cells of *Bacillus subtilis*. Antonie Van Leewenhoek *J. Microbiol. Serol*, **12**, 115-118 (1947).
8. LOOMIS, W. F., AND LIPMANN, F. Reversible inhibition of the coupling between phosphorylation and oxidation. *J. Biol. Chem.*, **173**, 807-808 (1948).
9. LOOMIS, W. F., AND LIPMANN, F. Inhibition of phosphorylation by azide in kidney homogenates. *J. Biol. Chem.*, **179**, 503-504 (1949).
10. PILLAI, R. K. Action of arsenate in glycolysis. *Biochem. J.*, **32**, 1961-1973 (1938).
11. POWELL, J. F. The sporulation and germination of a strain of *Bacillus megetherium*. *J. Gen. Microbiol.*, **5**, 993-1000 (1951).
12. PETERS, R. H., SINCLAIR, H. M., AND THOMPSON, R. H. S. An analysis of the inhibition of pyruvate oxidation by arsenicals in relation to the enzyme theory of visication. *Biochem. J.*, **40**, 516-524 (1946).
13. WYNNE, E. S., AND FOSTER, J. W. Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of quantitative methods. *J. Bact.*, **55**, 61-68 (1948).
14. WYNNE, E. S., AND FOSTER, J. W. Physiological studies on spore germination with special reference to *Clostridium botulinum*. II. Quantitative aspects of the germination process. *J. Bact.*, **55**, 69-73 (1948).

METHOD FOR BRAISING BEEF ROUND STEAKS[a]

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One of the bases for comparison of results obtained at different times and from different laboratories is the use of standard methods of procedure. In surveying the literature, and after discussing results obtained by a number of contractors and cooperators on the commercial grade beef work sponsored by the Bureau of Human Nutrition and Home Economics, it became apparent that no commonly accepted laboratory methods existed for meat cookery other than those for roasting meat. Accordingly, a project was initiated to investigate methods for braising beef round steaks cut one-inch thick. It was hoped that such a project would yield a method which could be used as a standard in this laboratory, and furnish a starting point for work in other laboratories to confirm or modify such methods.

PROCEDURE

Steaks were obtained from 3 pairs of beef rounds, 2 of commercial (animals I and II) and 1 of prime grade (animal III). After 7 to 10 days' aging at 2–3°C., the rounds were separated into muscles, and each muscle cut into 1-inch steaks. The steaks were wrapped individually in moisture-vapor-proof cellophane, frozen, and stored at −24°C. until needed. The muscles used and the number of one-inch steaks obtained from each muscle were: *semimembranosus*, 11–12; *semitendinosus*, 10–11; *adductor*, 5–6; *biceps femoris*, 12–16; *vastus lateralis*, 5–6; *rectus femoris*, 2–4; *gastrocnemius*, 6; *gracilis*, 4; *sartorius*, 2; *pectineus*, 3; *vastus medialis* and *intermedialis* (in one piece), 4. All muscles were cut across the grain, except the *gracilis*. The *gracilis* is a thin muscle with a large surface area. It was cut into quarters. The number of steaks obtained from each muscle varied with the size of the animal.

The variables considered were as follows:

1. Addition of 50 ml. of water after browning.
2. Use of a rack under the meat after browning.
3. Endpoint for cooking: internal temperature of 85°C., 98°C., 98°C., plus ½ hour additional cooking, 98°C. plus 1 hour additional cooking.
4. Browning: 475°F., 1 minute on each side; 400°F., 4 or 5 minutes on each side; 350°F., 7½ minutes on each side; none.
5. Oven temperature: 250°, 300°, 350°F.

The overall plan was designed to permit elimination of undesirable or impractical procedures as the work progressed. The variables were considered in units scaled to the number of paired steaks available from each pair of muscles. As each unit was completed, the data were analyzed, and the more desirable methods carried on to the next unit. Adjustments were made from animal to animal to include promising leads, or to compensate for differing number of steaks due to variations in the size of the animals.

The general cooking and testing procedure was as follows. Frozen steaks were thawed 24 hours in a refrigerator at 5°C. Cooking was done in heavy iron skillets, 4 inches in depth, with glass lids. The details of the cooking methods used are given in Table 1. If the steaks were to be browned, the skillets were preheated to the required temperature on an electric grill or the surface burners of an electric range. Griddle thermometers were used to check the temperature of the skillets. Subsequent

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TABLE 1

Description of cooking methods utilized

Series	Animal	Muscle	Cooking methods used	
			Constants	Variables
1	I, II	*Rectus femoris, vastus lateralis*	Browning: 400°F., 5 min. each side. Oven: 300°F. Endpoint: 98°C. + 1 hr.	Use of rack, no rack. Addition of 50 ml. water, no water (necessary to add 100 to 150 ml. water to each steak, to prevent burning with long cooking time at 300°F.)
	III	*Rectus femoris, gracilis, vastus medialis* and *intermedialis, gastrocnemius*	Browning: 475°F., 1 min. each side. Oven: 250°F. Endpoint: 98°C. + ½ hr.	Use of rack, no rack. Addition of 50 ml. water, no water.
2	I, II	*Gracilis, vastus lateralis*	Browning: 400°F., 5 min. per side. Oven: 300°F. No rack.	Endpoint: 85°C., 98°C., 98°C. + ½ hr., 98°C. + 1 hr. Addition of 50 ml. water, no water (necessary to add water to all samples cooked to 98° + ½ hr. and 98° + 1 hr.)
3	I	*Sartorius, pectineus, vastus medialis*	Browning: 400°F., 5 min. per side. Oven: 300°F. Water: 50 ml. added. Endpoint: 98°C. + ½ hr.	Use of rack, no rack.
4	I, II	*Biceps femoris*	No rack, 50 ml. water added.	Browning: 475°F., 1 min. per side; 400°F., 4 min. per side; 350°F., 7½ min. per side; none. Oven: 250°F., 350°F. Endpoint: 85°C., 98°, 98° + ½ hr., 98° + 1 hr.
	III	Same	Rack used, water added.	Browning: same. Oven: same. Endpoint: 85°C., 98°C., 98° + ½ hr.
5	I	*Semimembranosus*	No rack used, water added.	Browning: 475°F., 1 min./side; 400°F., 4 min./side; none. Oven: 250°F., 350°F. Endpoint: 85°C., 98°, 98° + ½ hr., 98° + 1 hr.

TABLE 1 (concluded)
Description of cooking methods utilized

Series	Animal	Muscle	Cooking methods used	
			Constants	Variables
	II	Same	Same	Browning: 400°F., 4 min./side; 350°F., 7½ min./side; none. Oven: same. Endpoint: same.
	III	Same	Rack used, water added.	Browning: 475°F., 1 min./side; 400°F., 4 min./side; 350°F., 7½ min./side; none. Oven: 250°F., 350°F. Endpoint: 85°C., 98°, 98° ½ hr.
6	I	*Semitendinosus*	No rack used, water added.	Browning: 475°F., 1 min./side; 400°F., 4 min./side; none. Oven: 250, 300, 350°F. Endpoint: 98°C., 98° + ½ hr.
	II	*Semitendinosus, adductor, gastrocnemius*	No rack used, water added, 300°F. oven.	Browning: 475°F., 1 min./side; 400°F., 4 min./side; 350°F., 7½ min./side; none. Endpoint: 85°C., 98°, 98° + ½ hr.
	III	*Semitendinosus*	Rack used, water added.	Browning: 475°F., 1 min./side; 400°F., 4 min./side; none. Oven: 250, 300, 350°F. Endpoint: 85°C., 98°, 98° + ½ hr.
7	I	*Adductor, gastrocnemius*	No rack used, water added.	Browning: 475°F., 1 min./side; 400°F., 4 min./side; none. Oven: 250°, 350°F. Endpoint: 60 min./lb., 100 min./lb.
8	III	*Adductor, vastus lateralis*	Rack used, water added.	Browning: 475°F., 1 min./side; 400°F., 4 min./side. Oven: 250, 300, 350°F. Endpoint: 98° + ½ hr.

cooking was done in gas ovens preheated to the indicated temperature, in covered skillets. When cooking was completed, the steaks were sliced and scored for aroma, appearance, flavor of lean, tenderness, juiciness, flavor of fat, and general acceptability. The scoring scale ranged from 10 *(extremely good)* to 1 *(extremely poor)*. The judges also recorded the number of chews required to masticate a standard-size bite. Data were recorded to permit calculation of cooking losses and temperature change with cooking time. Data on losses during freezing and thawing were recorded for steaks from two of the three animals.

RESULTS AND DISCUSSION

Freezing and thawing losses. Weights were recorded for animals II (commercial grade) and III (prime grade) so that losses due to freezing and thawing could be calculated. The average per cent losses for the steaks from each muscle were as follows:

	Animal II	Animal III
	%	%
Vastus lateralis	1.89	2.52
Rectus femoris	1.10	0.85
Gracilis	0.42	0.49
Biceps femoris	1.12	1.01
Semimembranosus	2.85	0.74
Semitendinosus	1.54	1.06
Vastus medialis and *intermedialis*		1.37
Gastrocnemius		0.60
Adductor		2.03
F-values		
between muscles within animal	10.40**	29.15**
between animals		15.62**

Addition of water. The first cooking variable considered was the addition of water just before the steaks were placed in the oven. The planned comparison was to be between steaks cooked without water and steaks to which 50 ml. of water were added before the pan was covered and placed in the oven. However, it was necessary to add water to all steaks cooked in a 300°F. oven to the endpoint of 98°C. plus ½ hour or 98°C. plus 1 hour. All steaks cooked to the latter endpoint required more than 50 ml. of water. The same cooking method was used for the cuts from animal II, to permit comparison of the data between the two animals within the same grade. However, since animal III was a different grade, it seemed advisable to shorten the browning procedure, lower the oven temperature, and use a shorter cooking time, to take advantage of the information already gained as to the most desirable method of braising a steak.

The average scores and total cooking losses for the units involving addition of water are listed in Table 2. With low oven temperature, and/or short cooking times, where it was possible to cook the steaks without added water, the steaks to which water was added were superior in palatability in nearly all cases. Also, the steaks with water added had slightly lower cooking losses than those cooked without water. With the other cooking methods, the addition of water was necessary to avoid burning the steaks. Therefore, it was decided to stipulate the addition of 50 ml. of water before the pans were placed in the oven.

TABLE 2

Data from Series 1 and 2 on addition of water as a variable

Animal	Series	Variable	Average Scores								Percent total cooking loss	
			Flavor of lean		Tenderness		Juiciness		General acceptability			
			Water added	No water	Water added	No water	Water added	No water	Water added	No water	Water added	No water
I—Commercial Grade	1	Rack used	6.0*	5.3*	5.7*	5.7*	3.2*	3.2*	4.5*	3.9*	46.0*	45.5*
		No rack used	6.3*	6.1*	5.7*	5.4*	3.7*	4.1*	4.4*	4.4*	47.7*	48.3*
	2	Endpoint 85°C.	7.1	6.9	6.0	5.5	6.2	6.0	6.7	6.0	35.6	36.5
		98°C.	6.5	6.3	5.4	5.5	5.7	5.1	6.0	5.8	38.9	39.8
		98°C. + ½ hr.	7.2	6.9*	5.9	5.9*	5.0	5.0*	5.6	6.2*	41.9	41.4*
		98°C. + 1 hr.	6.6*	6.9*	7.4*	7.1*	5.0*	4.8*	6.0*	6.3*	42.9*	42.2*
II—Commercial Grade	1	Rack used	6.5*	6.7*	5.7*	5.5*	4.5*	4.4*	5.0*	5.0*	46.5*	45.6*
		No rack used	6.3*	6.1*	5.0*	5.6*	4.7*	4.1*	5.0*	4.8*	45.4*	44.1*
	2	Endpoint 85°C.	6.8	6.2	4.8	3.7	6.0	5.4	5.7	4.7	36.2	37.6
		98°C.	6.2	6.5	4.4	3.8	5.6	4.7	5.4	4.7	38.6	39.7
		98°C. + ½ hr.	6.7	6.8*	5.6	5.4*	5.2	5.3*	5.3	5.3*	42.9	42.4*
		98°C. + 1 hr.	6.9*	6.2*	6.8*	5.6*	5.2*	4.7*	5.8*	5.1*	42.2*	40.5*
III—Prime Grade	1	Rack used	7.9	7.7	8.1	7.7	6.5	5.9	7.5	7.3	36.5	38.0
		No rack used	7.7	8.0	7.9	7.9	6.3	6.0	7.3	7.4	35.4	38.1

* Additional water required to prevent burning.

Use of rack. Most of the data referring to the use of a rack under the meat are listed in Table 2. In the first animal, the steaks cooked without a rack seemed to be more desirable, although omitting the rack increased the cooking losses slightly. On this basis, no rack was used for the remainder of the planned units for animal I. However, the first unit on animal II gave the opposite results—that is, the use of a rack gave a more desirable product. Therefore, a supplementary series (No. 3) was included to give additional data, using some of the extra muscles from animal I. In this series, the endpoint of 98°C. plus ½ hour was used, as the previous work on animal I had suggested that steaks cooked to 98°C. plus 1 hour were overcooked.

For series 3, the steaks cooked on a rack were more desirable (Table 3) than those cooked without a rack. However, it was decided to complete the tests on animal II without using a rack, to maintain the animals within grade comparison. In animal III, the differences attributable to use or non-use of a rack were not significant. In view of the results with animals I and II, it was decided to use a rack for the remaining tests on animal III.

TABLE 3

Data from Series 3 on advisability of using a rack (steaks from Animal I)

Muscle	Variable	Average score				Percent total cooking losses
		Flavor of lean	Tenderness	Juiciness	General acceptability	
Sartorius	Rack used	7.3	7.2	5.4	6.2	41.0
	No rack	7.1	7.2	6.0	6.1	33.3
Pectineus	Rack used	6.6	5.9	5.3	5.7	40.9
	No rack	6.0	5.3	4.4	5.0	40.3
Vastus Medialis	Rack used	6.5	5.1	4.9	5.1	41.8
	No rack	6.5	4.1	4.6	4.5	42.6

Endpoint. The 4 endpoints selected at the beginning of the study were internal temperatures of 85°C., 98°C., 98°C. plus ½ hour cooking after the temperature reached 98°C., and 98°C. plus 1 hour additional cooking. The averages and F-values obtained for these endpoints are listed in Table 4.

Longer cooking, up to 98°C. plus ½ hour, generally improved the flavor and increased the tenderness, but decreased the juiciness and increased the cooking losses. Often the palatability changes tended to cancel each other so that there were no significant differences among the general acceptability scores. Steaks cooked to 98°C. plus 1 hour were quite dry, and occasionally quite tough. Overall, 98°C. plus ½ hour seemed the endpoint most generally acceptable to our judges.

When the data from the first animal were calculated, the times in minutes per pound seemed to fall into two groups—about 60 minutes and about 100 minutes per pound. Accordingly some of the steaks from this animal were used in an extra series, No. 7, where the steaks were cooked either 60 or 100 minutes per pound. The results (Table 4) indicated that minutes per pound did not furnish a good basis for determining how long

TABLE 4

Averages and F-values for effect of end point

Series	Animal	Factor	End point				F-value
			85°C. Ave.	98°C. Ave.	98° + ½ hr. Ave.	98° + 1 hr. Ave.	
2	I, II	Flavor, lean	6.7	6.4	6.9	6.6	2.81
		Tenderness	5.0	4.8	5.7	6.7	8.22**
		Juiciness	5.9	5.3	5.1	4.9	1.28
		Gen'l Accept.	5.7	5.5	5.6	5.8	<1
		% Cooking loss	36.5	39.2	42.1	41.9	6.47**
4	I, II	Flavor, lean	6.4	6.3	6.7	6.7	1.34
		Tenderness	5.3	5.5	6.5	7.0	9.58**
		Juiciness	6.6	5.5	5.2	4.8	19.73**
		Gen'l Accept.	5.4	5.6	5.8	6.0	1.96
		% Cooking loss	34.5	39.6	42.0	44.4	65.18**
	III	Flavor, lean	7.3	7.4	8.0		18.49**
		Tenderness	7.1	6.9	8.2		25.76**
		Juiciness	6.7	6.0	5.7		6.27**
		Gen'l Accept.	7.0	6.7	7.5		10.72**
		% Cooking loss	28.2	34.6	39.0		90.63**
5	I	Flavor, lean	6.9	6.7	7.3	6.8	1.18
		Tenderness	5.7	5.6	6.7	5.7	1.69
		Juiciness	6.1	5.3	4.5	4.0	13.96**
		Gen'l Accept.	6.1	5.7	5.8	4.5	8.17**
		% Cooking loss	36.4	41.3	44.2	45.0	41.17**
	II	Flavor, lean	6.1	6.4	7.0	7.0	8.05**
		Tenderness	4.4	4.3	5.6	6.6	9.85**
		Juiciness	5.0	4.2	4.0	3.8	6.47**
		Gen'l Accept.	5.0	4.8	5.0	5.1	<1
		% Cooking loss	38.2	42.3	45.3	45.7	39.71**
	III	Flavor, lean	6.4	7.6	7.0		3.73
		Tenderness	5.7	7.1	6.9		7.70**
		Juiciness	5.1	5.2	4.7		1.02
		Gen'l Accept.	5.9	6.9	6.5		5.22**
		% Cooking loss	34.9	36.5	42.2		32.82**
6	I	Flavor, lean		7.3	7.5		1.14
		Tenderness		6.2	6.7		12.37**
		Juiciness		5.9	5.1		19.20**
		Gen'l Accept.		6.2	6.5		3.48
		% Cooking loss	34.2	39.0	43.0		14.61**
	II	Flavor, lean	6.2	6.7	6.9		10.64**
		Tenderness	6.3	6.0	6.4		<1
		Juiciness	6.0	5.1	4.6		16.50**
		Gen'l Accept.	5.9	5.9	6.1		<1
		% Cooking loss	34.8	41.4	43.8		161.86**
	III	Flavor, lean	7.1	7.3	7.8		2.93
		Tenderness	7.1	6.9	7.3		1.28
		Juiciness	5.6	5.0	4.9		10.90**
		Gen'l Accept.	6.9	6.9	7.1		<1
		% Cooking loss	31.9	37.1	40.0		248.52**

			100 min./lb.	60 min./lb.	F-value
7	I	Flavor, lean	7.1	6.7	5.39*
		Tenderness	6.1	6.2	<1
		Juiciness	4.3	5.0	5.11*
		Gen'l Accept.	5.5	5.4	<1
		% Cooking loss	41.4	37.8	11.17**

to braise one-inch steaks, since the general acceptability scores were among the lowest obtained for any of the cooking methods.

Browning temperature. The 4 sets of browning conditions used were: (1) browning each side 1 minute in a pan preheated to 475°F.; (2) browning each side 5 minutes in a pan preheated to 400°F. (time later reduced to 4 minutes each side because a few steaks scorched when browned 5 minutes); (3) browning each side 7½ minutes in a pan preheated to 350°F.; and (4) none—steak placed in cold pan and put directly into the oven. The average results and F-values from the series where browning was a variable are listed in Table 5.

Steaks browned at 475°F. received the highest scores for flavor and general acceptability. Those browned at 400°F. were generally highest in tenderness in the individual series, although those browned at 475°F. were slightly higher when all the data were averaged together. The steaks browned at 350°F. were the juiciest and had the lowest cooking losses. When considering the actual cooking procedure, it was decided that browning at 350°F. was not satisfactory because of length of time involved and because the steaks tended to leak moisture and were apt to "stew" rather than brown properly. The steaks cooked without browning did not brown well in the oven, so were unattractive in appearance. Therefore, 475°F. for 1 minute on each side was selected as the preferable method of browning one-inch steaks.

Oven temperature. The oven temperatures employed were 250°, 300°, and 350°F. The average scores, per cent cooking losses, F-values, and average cooking times in the oven are listed in Tables 6 and 7.

The oven temperature made very little difference in scores for flavor of lean, tenderness, juiciness and general acceptability, or in per cent cooking losses. Time required in the oven decreased with increasing oven temperature. In the cooking operation, it was found that the steaks cooked at the higher oven temperatures required careful watching to be sure that the pan did not boil dry and scorch. This was particularly true for the steaks cooked to 98°C. plus ½ hour or 98°C. plus 1 hour. Accordingly, it was decided to use 250°F. as the standard oven temperature for braising one-inch steaks.

Use of number of chews to determine tenderness. Determination of tenderness by the number of chews required to masticate a standard size sample of meat, as suggested by Lowe *(1)*, was included in the experiment. Some difficulty was experienced in cutting the same size pieces, since the different steaks were found to vary in thickness due to irregularities in cutting the raw steaks and to contraction of the steaks during cooking. Accordingly a table of widths and lengths of sample was developed, so that all pieces for chewing had the same surface area. The thickness of slice was controlled by cutting the samples on a mechanical slicer. The judges were asked to count the number of chews necessary to reduce the sample so that it could be swallowed without effort. The correlation coefficients between tenderness score and number of chews were: animal I, -0.87^{**}; animal II, -0.91^{**}; animal III, -0.82^{**}; and three animals together, -0.88^{**}. These high correlations suggest that counting the number of chews required to masticate a standard size sample of meat is a good

TABLE 5

Averages and F-values for browning temperature and time

Series	Animal	Factor	Browning conditions: 475°F. 1 min./side Ave.	400°F. 4–5 min./side Ave.	350°F. 7½ min./side Ave.	None Ave.	F. value
4	I, II	Flavor, lean	6.4	6.6	6.6	6.6	<1
		Tenderness	5.5	6.3	6.3	6.0	2.21
		Juiciness	5.1	5.2	6.0	5.7	5.26**
		Gen'l Accept.	5.3	5.8	5.9	5.8	2.27
		% Cooking loss	41.3	40.0	39.5	39.6	2.14
	III	Flavor, lean	8.1	7.7	7.2	7.3	12.54**
		Tenderness	7.6	6.9	7.1	7.9	8.53**
		Juiciness	6.8	6.2	5.8	5.6	5.59**
		Gen'l Accept.	7.5	6.9	6.7	7.2	6.54**
		% Cooking loss	35.7	32.9	31.3	35.8	11.08**
5	I	Flavor, lean	6.6	7.0		7.1	1.88
		Tenderness	5.8	6.1		5.9	<1
		Juiciness	4.9	4.6		5.4	2.87
		Gen'l Accept.	5.4	5.4		5.9	1.72
		% Cooking loss	41.7	41.8		41.5	<1
	II	Flavor, lean		6.7	6.8	6.5	1.93
		Tenderness		4.7	5.2	5.9	4.07*
		Juiciness		4.1	4.2	4.5	1.07
		Gen'l Accept.		4.8	4.9	5.2	1.13
		% Cooking loss		43.7	42.5	42.4	2.19
	III	Flavor, lean	7.5	7.0		6.5	2.55
		Tenderness	6.4	7.2		6.1	3.63
		Juiciness	4.7	5.3		4.9	1.67
		Gen'l Accept.	6.4	6.7		6.2	1.16
		% Cooking loss	40.1	35.5		38.2	5.87**
6	I	Flavor, lean	7.6	7.5		7.2	2.01
		Tenderness	6.7	6.5		6.2	4.28*
		Juiciness	5.4	5.6		5.4	<1
		Gen'l Accept.	6.5	6.6		5.9	8.11**
		% Cooking loss	40.4	40.4		39.6	<1
	II	Flavor, lean	6.7	6.6	6.6	6.4	<1
		Tenderness	6.4	6.4	6.3	5.8	1.14
		Juiciness	5.1	5.2	5.7	5.0	2.75
		Gen'l Accept.	6.1	5.8	6.1	5.8	1.55
		% Cooking loss	40.4	39.7	39.6	40.6	1.46
	III	Flavor, lean	7.9	7.3		7.0	4.91*
		Tenderness	7.2	7.3		6.8	1.53
		Juiciness	5.6	5.4		4.1	64.69**
		Gen'l Accept.	7.3	7.0		6.6	2.57
		% Cooking loss	36.9	35.5		38.6	30.85**
7	I	Flavor, lean	6.8	6.7		7.1	2.08
		Tenderness	6.1	5.9		6.5	4.59*
		Juiciness	3.9	4.4		5.7	11.85**
		Gen'l Accept.	5.3	5.3		5.8	2.74
		% Cooking loss	40.5	41.2		37.6	3.42
8	III	Flavor, lean	7.3	7.0			1.02
		Tenderness	7.0	7.0			0.0
		Juiciness	5.2	5.6			1.13
		Gen'l Accept.	6.3	6.2			<1
		% Cooking loss	41.9	42.5			<1

TABLE 6

Averages and F-values for oven temperatures

Series	Animal	Factor	Oven temperatures			F-value
			250°F. Ave.	300°F. Ave.	350°F. Ave.	
4	I, II	Flavor, lean	6.6		6.5	<1
		Tenderness	6.1		6.0	<1
		Juiciness	5.7		5.4	3.08
		Gen'l Accept.	5.8		5.6	1.53
		% Cooking loss	39.4		40.6	3.72
	III	Flavor, lean	7.4		7.8	10.41**
		Tenderness	7.3		7.4	<1
		Juiciness	6.1		6.2	<1
		Gen'l Accept.	7.0		7.2	2.36
		% Cooking loss	33.2		34.7	4.79*
5	I	Flavor, lean	6.8		7.0	<1
		Tenderness	6.1		5.8	<1
		Juiciness	5.0		5.0	<1
		Gen'l Accept.	5.6		5.5	<1
		% Cooking loss	42.1		41.3	1.96
	II	Flavor, lean	6.8		6.6	1.86
		Tenderness	5.4		5.1	1.03
		Juiciness	4.3		4.2	<1
		Gen'l Accept.	5.1		4.9	1.60
		% Cooking loss	42.8		42.9	<1
	III	Flavor, lean	6.8		7.1	<1
		Tenderness	6.5		6.6	<1
		Juiciness	4.9		5.0	<1
		Gen'l Accept.	6.3		6.6	<1
		% Cooking loss	37.8		38.2	<1
6	I	Flavor, lean	7.3	7.4	7.6	<1
		Tenderness	6.4	6.5	6.5	<1
		Juiciness	5.4	5.4	5.6	<1
		Gen'l Accept.	6.2	6.3	6.5	<1
		% Cooking loss	39.7	40.3	40.2	<1
	III	Flavor, lean	7.5	7.6	7.3	<1
		Tenderness	7.1	7.0	7.1	<1
		Juiciness	5.1	5.0	5.3	2.43
		Gen'l Accept.	7.1	6.9	7.0	<1
		% Cooking loss	37.6	37.4	36.3	<1
7	I	Flavor, lean	6.7	7.0		4.10
		Tenderness	6.0	6.3		2.71
		Juiciness	4.7	4.6		<1
		Gen'l Accept.	5.3	5.6		2.92
		% Cooking loss	40.2	39.3		<1
8	III	Flavor, lean	7.2	6.9	7.2	<1
		Tenderness	7.2	6.8	6.9	<1
		Juiciness	5.5	5.3	5.4	<1
		Gen'l Accept.	6.3	6.1	6.3	<1
		% Cooking loss	41.1	42.7	43.0	1.72

TABLE 7

Average time in oven required to reach indicated end point

Browning conditions	End point	Average cooking time in oven (minutes)		
		250°F.	300°F.	350°F.
475°F., 1 min. per side	85°C.	21.8	14.7	13.6
	98°C.	38.6	26.3	27.3
	98°C. + ½ hr.	66.5	56.3	53.6
	98° + 1 hr.	107.0		81.7
400°F., 4–5 min. per side	85°C.	26.3	16.0	14.7
	98°C.	46.1	21.0	21.7
	98°C. + ½ hr.	63.8	58.5	51.4
	98° + 1 hr.	101.7		83.3
350°F., 7½ min. per side	85°C.	28.2	14.5	14.2
	98°C.	43.8	25.0	23.3
	98°C. + ½ hr.	73.2	53.0	51.4
	98° + 1 hr.	99.0		85.3
None	85°C.	48.3	29.0	27.4
	98°C.	56.5	40.0	34.4
	98°C. + ½ hr.	92.9	68.3	68.1
	98° + 1 hr.	130.3		96.8

method of determining tenderness. The judges felt that it helped them, as they didn't have to remember a tenderness standard.

SUMMARY AND CONCLUSIONS

An investigation was made in an attempt to establish a standard laboratory procedure for braising one-inch round steaks. Paired steaks from the individual muscles of the rounds of three beef animals were used. Two animals were commercial grade, the third prime grade.

The cooking procedures investigated included:

1. Addition of 50 ml. water.
2. Use of a rack under the steak.
3. Browning conditions: 475°F. for 1 minute on each side; 400°F. for 4 or 5 minutes on each side; 350°F. for 7½ minutes on each side; none.
4. Endpoint: 85°C. internal temperature; 98°C.; 98°C. plus ½ hour cooking after reaching 98°; 98°C. plus 1 hour cooking after reaching 98°.
5. Oven temperature: 250°F.; 300°F.; 350°F.

Data were collected on palatability factors and per cent cooking losses for all steaks. Losses during freezing and thawing were recorded for steaks from animals II and III.

The average losses due to freezing and thawing ranged from 0.42% for the *gracilis* to 2.85% for the *semimembranosus*. The differences between muscles within the animals, and between animals (one commercial, one prime grade) were highly significant.

Correlation coefficients indicated that number of chews required to masticate a sample of standard surface area and thickness was very effective as a means of measuring tenderness.

On the basis of the data obtained, the following procedure is suggested as a standard method for braising one-inch steaks.

1. Brown the steak at 475°F. for 1 minute on each side in a heavy iron skillet.
2. Place a rack with ½-inch legs under the steak.
3. Add 50 ml. water and cover the pan.
4. Place in an over preheated to 250°F.
5. Cook ½ hour longer than required to bring the internal temperature of the steak to 98°C.

It is recognized that work done in one laboratory is not sufficient for establishment of widely applicable standard methods. Comments on this method from other laboratories will be welcomed.

LITERATURE CITED

1. Lowe, B. *Experimental Cookery.* 4th ed., 1955. John Wiley and Sons, Inc.

THE MICROBIOLOGICAL DETERMINATION OF THE ESSENTIAL AMINO ACIDS IN FISH PROTEIN[a]

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(Manuscript received August 1, 1955)

Microbiological technique has proved valuable in determining the composition of various proteins. The purpose of this investigation was to determine the exact amounts of the ten essential amino acids (lysine, arginine, histidine, leucine, isoleucine, valine, threonine, methionine, phenylalanine and tryptophan) in three varieties of raw fish, i.e., shad, haddock and pomfret.

MATERIALS AND METHODS

Materials. With regard to the materials used, the following details are pertinent. Shad *(Alosa sapidissima)*, a highly valued fish in the United States, is available in huge quantities in the rainy season in various parts of India. The Indian equivalent of shad is called *Clupea ilisha*. Haddock *(Melanogrammus aeglefinus)*, the fish most commonly consumed in New England, may be obtained fresh throughout the year. Pomfret *(Stromateus cinereus)* is used as food in great quantities in Bombay. For these experiments pomfret was vacuum dried in India and imported via air shipment.

Methods. The fresh fillets of shad and haddock were ground to a slurry in a Waring blender with the addition of a small amount of water. The slurry was spread in thin layers on aluminum pans and dried in a vacuum belt drier at 2 mm. pressure and 15°F. The dried fish muscle was finely powdered in a Wiley Mill, then sieved and stored in the refrigerator in a glass-stoppered bottle.

The proximate composition of the three ether-extracted fish proteins was determined according to the methods recommended by the Association of Official Agricultural Chemists.

The composition of these three fish proteins, expressed as percentage of the total material, was as follows:

	Moisture	Ash	Crude Protein N × 6.25
Shad (Sample from tunnel-dried material)	6.67	5.4	88.56
Haddock (Sample from tunnel-dried material)	7.91	4.24	90.31
as sent from India)	8.40	8.05	84.37
Pomfret (Sample from vacuum-dried material			

Fat was estimated on the powdered sample and expressed as percentage of the total material, i.e.

Shad	40.20%
Haddock	0.44%
Pomfret	0.62%

Assay determination of essential amino acids. Leuconostoc mesenteroides P-60[c] was used as the test organism in all the assays carried out in these experiments.

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[c] The culture (M. 8042) was obtained from the American Type Culture Collection, 2029 M. Street, N. W., Washington, D. C.

The medium developed by Dunn and co-workers was used *(1)*. When the growth requirements of an organism have been ascertained, it becomes possible to devise synthetic media of known composition that are deficient in a single substance only. Then the response of the organism to additions of that substance may be employed for its quantitative estimation and may be determined either turbidimetrically or by titrating the acid produced.

A sample (1–1.5 g.) of dried fish muscle was mixed with 50–75 ml. of 2 N HCl in a 100-ml. Erlenmeyer flask. The flask was kept in an autoclave at 15 lbs. pressure from 5 to 6 hours. The mouth of the flask was covered with an inverted beaker. After hydrolysis, the flask was cooled and the contents were diluted with about 15 ml. of water and filtered through a Whatman filter paper (No. 4) into a 100-ml. volumetric flask. The Erlenmeyer flask and the filter paper were washed several times with small quantities of distilled water. The contents of the volumetric flask were then made up to the mark with distilled water and mixed thoroughly. This stock hydrolysate was stored in the refrigerator in tightly-stoppered brown glass bottles.

Each time an amino acid was determined, aliquots of stock hydrolysate were pipetted into 100-ml. volumetric flasks. The material was diluted with distilled water, the pH of the diluted hydrolysate was adjusted with NaOH, and it was made up to volume.

Alkaline hydrolysates made with 3 to 5 N NaOH were used for tryptophan determination. Tubes were set up as follows:

Tube number	Basal medium	Test solution, ml.	Distilled water, ml.
1	2	0.1	0.9
2	2	0.2	0.8
3	2	0.3	0.7
4	2	0.4	0.6
5	2	0.5	0.5
6	2	0.6	0.4

After thorough mixing of the contents, the tubes were covered with a clean towel and autoclaved at 15 lbs. pressure for 10 minutes. They were cooled to 37–40°C., aseptically inoculated with one drop of inoculum and incubated for 72 hours at 37°C.

The contents of each tube were titrated with 0.1 N NaOH, and 0.3 ml. (6 drops) of 0.04% bromothymol blue was used as the indicator.

A standard curve was constructed relating the response of the organism to the concentration of the amino acids. The amount of amino acid present in the unknown sample was determined by interpolation of the response onto this standard curve. The linear portion of these curves has been used for the calculation of the results. The final result taken was an average of values calculated from several levels on the standard curve.

Recovery experiments were carried out for each assay to establish the validity of the assay results.

The amino acid content of fish muscle as determined by microbiological analysis is shown in Table 1.

TABLE 1

Essential amino acid content of raw fish muscle[1]

Amino acid	Shad	Haddock	Pomfret
Lysine	9.82	8.56	10.82
Arginine	5.69	5.68	5.41
Histidine	2.28	1.88	1.90
Leucine	7.65	7.52	7.32
Isoleucine	4.99	5.41	5.03
Valine	6.17	5.64	4.02
Threonine	4.05	4.23	4.44
Methionine	2.82	2.78	2.56
Phenylalanine	3.65	3.67	3.19
Tryptophan	1.10	0.94	0.87

[1] The amino acid values are given as grams amino acid yielded by 100 grams anhydrous ash-free protein.

A sample of the ether-extracted shad flesh was analysed independently in another laboratory[4] for amino acid content by microbiological methods, and the findings, together with our results, are shown in Table 2. It will be noted that the values show small differences such as can be accounted for within the errors of the experimental methods.

RESULTS AND DISCUSSION

The essential amino acid composition of the 3 varieties of fish as determined by microbiological methods was found to be comparable to that reported for beef and other animal proteins *(2)*. Table 3 gives the essential amino acid composition of shad, pork, and beef muscle (grams of amino acid per 16 g. of nitrogen).

A deficiency of lysine, methionine, and tryptophan is often found in foods of vegetable origin. Further, the nutritive quality of a protein is dependent to a considerable extent upon these 3 amino acids. These findings show that the 3 species of fish under examination have proved

[4]Professor H. Williams and his colleagues of the Department of Biochemistry and Nutrition, Cornell University, kindly supplied the analysis.

TABLE 2

Essential amino acid composition of shad muscle[1]

Amino acid	(a) Authors' findings	(b) Authors' findings	Findings by Williams *et al.*[2]
	(a)	(b)	
Lysine	8.80	8.40	9.63
Arginine	5.08	4.92	5.42
Histidine	2.00	2.00	2.06
Leucine	6.70	6.60	6.60
Isoleucine	4.34	4.39	4.89
Valine	5.50	5.30	6.07
Threonine	3.45	3.63	3.81
Phenylalanine	3.20	3.20	3.50
Methionine	2.50	2.43	2.46
Tryptophan	0.92	1.01	1.07

[1] The amino acid values are given as grams amino acid yielded by 100 grams protein uncorrected for moisture and ash.
[2] Unpublished data; kindness of Dr. H. Williams and colleagues, Cornell University.

TABLE 3

Essential amino acid composition[1] of fish muscle, pork muscle, and beef muscle *(2)*

Amino acid	Shad muscle	Pork muscle	Beef muscle
Lysine	9.82	8.50	8.70
Arginine	5.69	6.30	6.10
Histidine	2.28	3.30	3.60
Leucine	7.65	7.80	7.80
Isoleucine	4.99	4.80	5.00
Valine	6.17	5.20	5.20
Threonine	4.05	4.30	4.50
Phenylalanine	2.82	3.80	3.80
Methionine	3.65	2.50	2.70
Tryptophan	1.10	0.90	1.00

[1] The amino acid values are given as grams amino acid yielded by 100 grams anhydrous ash-free protein.

to be an excellent source of lysine and good sources of tryptophan and methionine.

The results of these experiments showing fish protein to be of high nutritive value are of special importance with reference to the nutritional problems of nations like India that suffer from a shortage of protein foods but do have access to deep-sea fishing.

LITERATURE CITED

1. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B. Investigations of amino acids, peptides, and proteins. XVIII. The amino acid requirements of *Leuconostoc mesenteroides* P-60. *J. Biol. Chem.*, **156**, 703–713 (1944).

2. Neilands, J. B., Sirny, R. J., Sohljell, I., Strong, F. M., and Elvehjem, C. A. The nutritive value of canned foods. II. Amino acid content of fish and meat products. *J. Nutrition*, **39**, 187–202 (1949).

EVALUATION OF AMINO ACIDS IN FISH PROCESSED BY VARIOUS METHODS[a]

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(Manuscript received August 1, 1955)

With the increasing popularity of processed fish among both urban and rural populations, the question as to whether or not consumers of processed fish are deriving the full benefit of a protein food has acquired added significance. The present study was undertaken to determine whether commercial canning and dehydration have any destructive effect on the essential amino acids of shad and haddock.

MATERIALS AND METHODS

Materials. Shad *(Alosa sapidissima)* and haddock *(Melanogrammus aeglefinus)* were used for this investigation.

Dehydration. Fresh fillets of shad meat were cut into thin strips ½ inch long, ⅛th inch thick, and ⅛th inch wide, and these strips were cooked for 10 minutes at 5 pounds' pressure. The cooked pieces were next dipped in 0.05% l-ascorbic acid solution for one minute. The preheated fish was then dried in a vacuum belt drier *(3)* under the following conditions: air temperature, 190°F. for first 300 minutes and 170°F. for the next 250 minutes; relative humidity, 10%; air velocity, 450 feet per minute. At the end of dehydration, the product was allowed to cool uniformly.

The above procedure was also used for dehydration of haddock fillets, with one exception; namely, that the cooked pieces were not dipped in the ascorbic acid solution as the development of rancidity was not a problem in the dehydration of haddock fillets.

Canning procedure. The main problem in canning shad meat is its soft meat and oily nature. To produce the desirable hardness in texture, various salt solutions were tried. Treatment with 60° salometer sodium chloride brine gave the most desirable texture. Exhaustion for 40 minutes in flowing steam removed much of the oil and improved the odor.

Frozen shad was thawed, scaled, and eviscerated. Head, tail, and fins were first removed, and the fish was then filleted. Fillets were treated with 60° salometer sodium chloride brine for 2 hours. Brine-treated fillets were packed by hand in No. 1 cans.

Fresh haddock was scaled, eviscerated, and cooked in flowing steam for 10 minutes. The muscle was then flaked, and the flakes were packed by hand in No. 1 cans. Five grams of sodium chloride were added per pound of flakes.

The cans of fish were exhausted for 40 minutes in flowing steam, and the sealed cans were processed for 60 minutes under a steam pressure of 10 lbs. p.s.i.g.

Microbiological assay. The dehydrated fish, after ethyl ether extraction, and the canned fish, after being dried in the vacuum belt drier at 2-mm. pressure and 15°F. were subjected to microbiological assay for determination of essential amino acids. The technique employed was similar to that used by Proctor and Lahiry *(2)*. *Leuconostoc mesenteroides* P-60 was used as the test organism in all assays. Before a protein sample can be analyzed microbiologically by use of common lactic acid organisms, the protein must be hydrolysed to free the amino acids. In this study 2 N HCl was used as the hydrolysing agent. The materials were heated for 6 hours at 15-lb. pressure (121°C.). Aliquots of the hydrolysates were used for the estimation of the essential amino acids.

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Identical values were obtained in duplicate and even triplicate experiments for each of the ten essential amino acids determined. It may be concluded, therefore, that the microbiological procedure employed was adequate.

RESULTS AND DISCUSSION

From the experimental data given in Tables 1 and 2 it is apparent that with shad and with haddock the amino acid contents did not differ significantly whether the fish was raw, dehydrated, or canned. It may be concluded that the common methods of heat processing did not affect the amino acid contents of these fish. These findings are in close agreement with those of Neilands *et al. (1)*, who measured the amino acid contents of raw and canned Atlantic fish flakes (cod and haddock) and other species of fish.

TABLE 1

Amino acid content of raw and heat processed shad[1]

Amino acid	Shad (raw)	Shad (dehydrated)	Shad (canned)
Lysine	9.82	9.45	9.03
Arginine	5.69	5.65	5.24
Histidine	2.28	2.17	1.87
Leucine	7.65	7.57	7.53
Isoleucine	4.99	5.20	4.57
Valine	6.17	5.84	6.72
Threonine	4.05	4.22	4.05
Methionine	2.82	2.71	3.01
Phenylalanine	3.65	3.39	3.76
Tryptophan	1.10	0.93	0.84

[1] The amino acid values are given as grams amino acid yielded by 100 grams anhydrous ash-free protein.

TABLE 2

Amino acid content of raw and heat processed haddock[1]

Amino acid	Haddock (raw)	Haddock (dehydrated)	Haddock (canned)
Lysine	8.56	9.15	8.14
Arginine	5.68	5.32	5.20
Histidine	1.88	1.69	1.51
Leucine	7.52	7.44	7.62
Isoleucine	5.41	5.11	5.24
Valine	5.64	5.20	5.11
Threonine	4.23	4.20	4.37
Methionine	2.78	2.60	3.12
Phenylalanine	3.67	3.21	3.68
Tryptophan	0.94	0.89	1.10

[1] The amino acid values are given as grams amino acid yielded by 100 grams anhydrous ash-free protein.

LITERATURE CITED

1. Neilands, J. B., Sirny, R. J., Sohljell, I., Strong, F. M., and Elvehjem, C. A. The nutritive value of canned foods. II. Amino acid content of fish and meat products. *J. Nutrition*, 39, 187–202 (1949).
2. Proctor, B. E., and Lahiry, N. L. The microbiological determination of the essential amino acids in fish protein. *Food Research*, 21, (this issue).
3. Sluder, J. C., Olsen, R. W., and Kenyon, E. M. A method for the production of dry powdered orange juice. *Food Technol.*, 1, 85–94 (1947).

TEXTURE CHANGES DURING THE DARK-RIPE PROCESSING OF OLIVES

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In addition to its role in the production of new flavors, pickling in brine often is associated with pronounced alterations in the texture of the brined product. Characteristically, softening has been observed frequently, and the relationship of such softening to the breakdown of pectic materials of the cell walls has been well documented *(4, 6, 11, 15)*.

On the other hand, some evidence has accumulated which suggests that during the normal brining process a firming effect may predominate. The crisp texture of sauerkraut, of cucumber pickles, and of Spanish-style olives is well-known. In brined cherries, McCready and McComb *(9)* note a definite "firming reaction" during the brining process. Moreover, olives made by the "dark-ripe" method seem to be less susceptible to softening during heat treatment than fresh, un-brined ones.

Besides the brining operation, the dark-ripe processing of olives *(2)* involves treatment with lye solutions (with attendant aeration for color production), leaching of the lye from the olives, salting in 2.5% NaCl filling solution, and retorting at 240°-250°F. These treatments undoubtedly affect the final texture of the canned olive. The present investigation has been undertaken to ascertain the respective contributions of each processing phase to the textural quality of the finished product.

MATERIALS AND METHODS

The raw materials of the experiments were Ascolano and Sevillano olives, harvested at various maturity stages during 1951, 1952, and 1953. Inasmuch as the experimental treatments varied from year to year, these are best considered in chronological order.

The 1951 experiments. Ascolano olives of a single maturity were harvested from one tree at Davis (Yolo Co.), California, for the initial experimentation. The olives were put in solutions in covered, 2-quart, canning-type glass jars. Some jars of fruit were permitted to ferment normally in 40° salometer (10%) brine with an added "starter" inoculum of *Lactobacillus plantarum*. In addition, sterile solutions were prepared with 5% formalin in distilled water to evaluate the effects of certain chemicals. These chemicals were as follows: sodium chloride (0, 0.01, 0.1, 1.0, and 10.0%); lactic acid (0, 0.01, 0.1, 1.0, and 10.0%), and calcium chloride (with calcium ion concentrations of 0, 10, 100, 1,000, and 10,000 p.p.m.). Sodium fluoride (0.4%) and sodium benzoate (0.2%) had to be added later to some of these solutions in order to inhibit microbial activity.

For texture evaluation, olives were pitted with a laboratory cork-borer, cut in half longitudinally, and placed in the test-box of a Tenderometer.[a] Texture measurements were made on raw and cooked (*i.e.*, 280 g. pitted olives boiled 5 minutes in 500 ml. distilled water) olives initially and at weekly intervals during the 3-month period of holding. At the end of 3 months, the texture of olives held in the sterile solutions with various chemicals was also measured. Histological specimens in paraffin were prepared for each type of treatment according to standard microtechnical methods *(5)*. These were cut at 10 μ thickness and stained with aqueous methylene blue solution (1/5,000) to indicate changes in amount of pectic substances in the cell walls.

[a] Food Machinery Corporation.

The 1952 experiments. In 1952, three different harvestings of Ascolano olives were made in the Davis area, and three different harvestings of Sevillano olives were shipped to Davis from Lindsay, California. At the earliest harvesting, the fruit was quite firm and green in color; at the second one, the color was yellowish-green and the flesh firm; the third sample was yellowish to red, and the flesh was becoming soft.

Most olives were put in jars to ferment in a 24° salometer (6%) brine (made from C.P. grade NaCl). As a replication of part of the 1951 experiments, some Ascolano olives of the second collection were put in various solutions which were kept sterile by the addition of toluene. These were composed as follows: distilled water, sodium chloride (1, 2, 4, 6, and 8%), sucrose (2, 4, 8, 12, and 16%), and calcium chloride (calcium ion concentrations of 100, 200, 400, 600 and 1,000 p.p.m.). Some normally fermenting solutions of both varieties also had calcium ion added in concentrations of 200 and 600 p.p.m., respectively.

Samples were drawn from the normally fermenting olives for strength testing, in the raw and cooked condition, with the Christel Texturemeter.[b] In addition, aliquots of the olives were processed by the dark-ripe method: immersed in 1% NaOH solution (with intermittent aeration), then leached, salted in 2.5% NaCl, canned, and placed in a retort at 242°F. for 1 hour. Some olives were leached in distilled and some in tap water (total hardness as $CaCO_3$-292 p.p.m.). Likewise, the NaCl filling solution was made in two parts: with or without added $CaCl_2$ (200 p.p.m. calcium ion). At each stage in processing, texture measurements were made and histological samples taken over a period of 12 or more weeks.

The 1953 experiments. Samples of two different maturities of Ascolano and Sevillano olives were sent to Davis from the Lindsay area. These were put in 2-quart jars to ferment normally in 6, 8, and 10% NaCl solutions. During the twelve-week storage period the texture of raw and cooked olives, principally from the 6% brine, was measured periodically with the Texturemeter, and samples were canned by the procedure outlined above.

Samples from the brine storage were also put up at 0, 2, 4, 8, and 12 week intervals in boiling 95% ethanol in a ratio of 2 parts of alcohol to 1 part of sample. Assays of these samples were made following blending of the alcohol-olive mixture, filtration on a Buchner funnel, ethyl ether-extraction for 24 hours in a Soxhlet apparatus, drying under vacuum at 60°C. for 24 hours, and weighing.

The analyses were devoted principally to exploring the nature of the pectic materials. Fractionation of the sample into three parts was performed as follows:

1. Homogenization with 9 parts of distilled water in a Waring blender, followed by a two-hour mechanical agitation of the slurry under toluene. The slurry was centrifuged and the supernatant saved. Another two-hour extraction was performed, and the second supernatant was combined with the first as the water-soluble fraction.

2. A second extraction was made with 0.05% Versene solution (adjusted to pH 7 with acetic acid) at room temperature. This extraction was repeated after centrifugation, and the two supernatants were combined into the Versene-soluble fraction.

3. The residue was heated in 0.05N HCl for 2 hours at 95°C. After centrifugation, the supernatant was used as the HCl-soluble fraction.

Aliquots from the different fractions were treated with twice their volumes of 95% ethanol. The precipitate was dried *in vacuo* for 24 hours at 60°C. and weighed, to give the alcohol-insoluble material. This precipitate was re-dissolved in water and an aliquot used to determine its methoxyl content, according to the method of Owens *et al. (12)*. Anhydrogalacturonic acid was determined on a second aliquot *(7)*. (Also, total anhydrogalacturonic acid was determined on the residue remaining after ether extraction, by the method of McCready and McComb, *7.)* Calcium determinations were made in the manner described by Bettelheim and Sterling *(1)*. Similarly, the intrinsic viscosity was determined according to the procedures described earlier *(1)*, but the concentrations used here were based on the alcohol-insoluble material.[c]

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[b] Mention of equipment or other items by brand name does not constitute an indorsement by this journal over items in the same category manufactured under other brand names.

[c] The author is indebted to F. A. Bettelheim for the determinations of pectic materials in this study.

RESULTS

Texture changes. *Effect of time in normal holding brine.* In the raw olives the course of texture changes appeared to be dependent on the relative maturity of the olive. Older olives maintained or even increased their original strength ratings (Figures 1, 2),

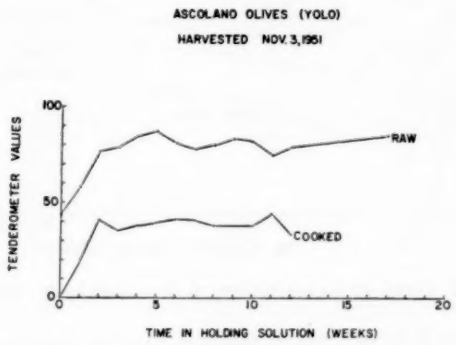

Figure 1. Course of texture changes in olives held in 10% NaCl brine.

particularly in the Ascolano variety. (In the Sevillano variety, there were occasional samples with a slight decrease in strength during the time held in brine storage, Table 1). However, in both varieties the younger olives suffered a diminution in shear strength as they remained in the holding brine (Figure 2; Table 1).

When the brined olive was cooked (as an approximation to the heating effect during processing), the results in all cases indicated a marked firming effect as the result of standing in brine solutions. (Figures 1, 2; Table 1). Occasional decreases in strength were noted in some jars, but these did not develop until after twelve weeks in the brine.

Except in the mature Ascolano olives, the strength of the canned product generally increased with time in the holding brine. Maximum values were attained within 6-8 weeks, with subsequent declines in shear strength (Table 2).

Effect of variations in sterile holding brine. Table 3 presents the results of studies with raw Ascolano olives in 1951. The firming effect of higher concentrations of NaCl and $CaCl_2$ is evident here. Likewise, the tenderizing role of lactic acid becomes apparent at relatively high concentrations. In 1952, the experiment was repeated in part. From

TABLE 1

Texturemeter values of 1953 olives held in 6% NaCl solution

Variety	Maturity	Treatment	Time in holding brine (weeks)			
			0	4	8	12
Ascolano	Firm-green	Raw	121.6	89.6	85.7	78.5
		Cooked	13.4	28.0	47.3	38.0
Sevillano	Firm-green	Raw	126.4	98.8	95.3	83.0
		Cooked	24.6	33.8	51.0	52.0
Ascolano	Soft-greenish-yellow	Raw	46.0	59.8	57.0	.....[1]
		Cooked	0.2	6.4	17.6	30.0
Sevillano	Soft-greenish-yellow	Raw	89.2	81.4	70.6	.....[1]
		Cooked	22.0	33.2	33.4	53.3

[1] Insufficient material for all determinations.

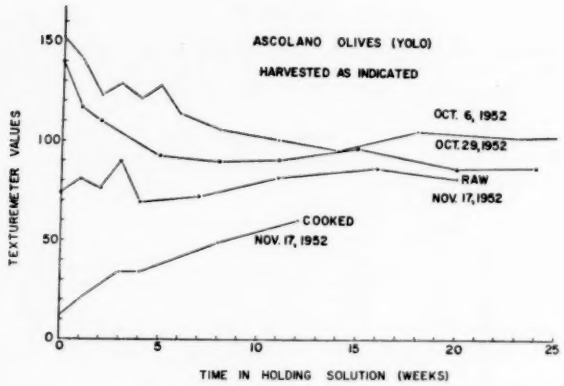

Figure 2. Course of texture changes in olives held in 6% NaCl brine.

the results given in Table 4, it may be seen that, as in the preceding year, higher concentrations of NaCl and $CaCl_2$ are related with increasing firmness of the olives. The relationship of sucrose concentration with textural strength is essentially similar to that of the salts.

(Insufficient material from variations in NaCl and in $CaCl_2$ content was available for adequate cooking and canning tests. The few results obtained showed a slight positive relationship between concentration of these salts in the brine and the strength of cooked or of canned olives.)

Effect of successive steps in processing. When distilled water is used throughout the the holding brine, lye, and salt solutions and for leaching the lye from the olives, it is clear from Table 5 that (a) the lye treatment and leaching cause a marked reduction in tissue strength, (b) the immersion in 2.5% NaCl solution before canning firms the olives appreciably, and (c) the retorting operation again causes some loss in olive firmness.

The role of calcium and other polyvalent actions is readily seen in the variations due to the use of tap water or of distilled water in leaching and the use of the NaCl filling solutions made with or without calcium ion. The decrease in mechanical strength in processing is greatest when distilled water is used throughout and least when leaching and salting occur with water in which calcium ion is present.

Histological aspects. Like most drupaceous fruits, the olive has a thin "skin," composed of a heavily cutinized epidermal layer and two underlying layers of thick-walled collenchymatous cells. The walls of these latter cells are richly furnished with pectic materials. Between the "skin" and the olive pit lies the bulk of fleshy tissue, composed principally of large, isodiametric, and thin-walled parenchyma cells. Branched sclereid

TABLE 2

Texturemeter values of processed 1952 and 1953 olives after holding in 6% NaCl solution (overall average)

Time in holding brine (weeks)	Strength of canned olives (texturemeter reading)
0	30.3
2	34.0
3	40.0
4	41.0
5	44.0
6	45.0
8	43.0
12	34.5

TABLE 3

Tenderometer values for holding solution variants with raw 1951 Ascolano olives (after 12 weeks)

Treatment		Tenderometer reading
Control (dist. water)		61.0
NaCl (%)	0.01	60.4
	0.1	60.4
	1.0	63.9
	10.0	83.2
Lactic acid (%)	0.01	60.0
	0.1	59.4
	1.0	61.0
	10.0	52.0
$CaCl_2$ (Ca^{++}p.p.m.)	10	66.0
	100	64.3
	1,000	66.9
	10,000	84.5

cells occur singly and randomly throughout the parenchyma tissue. These cells have thick walls which are heavily lignified. However, because of their separation from each other, they are not as perceptible to the tongue and teeth as are the clusters of sclereids in the flesh of the pear *(14)*. Also throughout the flesh are scattered the narrow strands of vascular tissue.

Figure 3 presents the major features of organization of the outer portion of an olive fruit. The methylene blue stain gives a deep purple color to local concentrations of pectic materials, such as occur in the collenchyma cells just below the epidermis. Following 26 weeks of brining (Figure 4), it is apparent that few noticeable changes have occurred in the cell wall material. There is a slight wrinkling in the walls of the parenchyma cells, probably due to the plasmolytic action of the holding brine. (This effect is also expressed in the shrunken protoplasts of these cells.) No staining change has occurred. Possibly the use of the newer pectin-staining technique of McCready and

TABLE 4

Texturemeter values for holding solution variants with raw 1952 Ascolano olives (Averages of 14 weeks)

Treatment		Texturemeter reading
Control (dist. water)		97.0
NaCl (%)	1.0	99.5
	2.0	102.8
	4.0	103.5
	6.0	101.3
	8.0	106.8
$CaCl_2$ (Ca^{++}p.p.m.)	100	99.3
	200	98.8
	400	101.8
	600	101.8
	1,000	104.0
Sucrose (%)	2.0	97.8
	4.0	101.5
	8.0	104.8
	12.0	106.5
	16.0	110.8

TABLE 5

Texturemeter values for 1952 olives at various stages in dark-ripe processing

Sample	Raw	Process				Retorting
		1% NaOH + leaching in		2.5% NaCl in		
		Distilled water	Tap water	Distilled water	200 p.p.m. Ca^{++}	
Average[1]	102.9	42.6		58.7		37.8
Ascolano samples	74.2	40.8		52.3		17.0
					59.8	37.0
			62.6	58.3		40.0
					61.2	49.7

[1] Average for both varieties.

Reeve *(10)* might have been illuminating, since changes in the extent of pectin esterification would perhaps have been indicated.

The cooking of olives, as well as their lye treatment, was definitely evidenced by a loss of stainability of the cell walls (Figures 5, 6). However no other step in processing could be clearly denoted by changes in methylene blue stainability in this study.

Analysis of pectic materials. No consistent relationship was found between the intrinsic viscosity of any pectin fraction (Table 6) and mechanical measures of tissue firmness. Similarly, no relationship was indicated between tissue firmness and the total amount of pectic substances. However, the principal determinations which did indicate a definite trend were the total calcium (calculated as percentage of the anhydrogalacturonic acid) and the methoxyl content of the Versene-soluble fraction.[4] Table 6 shows the average tendency for calcium content to increase during brine storage and the corresponding tendency for methoxyl content to decrease from the initial values. Note that these trends are much more definite in the older olives. (The 12-week determination of methoxyl content may be larger because of sampling or experimental errors.) The total anhydrogalacturonic acid content remained more or less constant throughout, at an average value of 0.63% of the fresh weight of the olive.

DISCUSSION

The evident increase in firmness of raw olives as they remain in holding brine may involve two relationships. On the one hand, as pointed out recently by McCready and McComb *(9)*, there is good evidence that the natural pectic esterase of the fruit (which is not inactivated during brining) acts slowly to de-methoxylate the pectic materials, with a consequent increase in their carboxyl content. The free acid groups thus produced can react with any available cations, which are usually in large supply in the cell sap. (For 100 g. of olives, 1 mg. of calcium ion would be sufficient for the neutralization of theoretically completely de-esterified pectic material.) The firming action of calcium pectate is already well known *(6)*.

Increasing firmness of the raw older olive as against the decreasing firmness of the raw younger olive also can be related to the esterase content. Probably, as indicated in Table 6, there is a relatively increased activity or increased supply of pectic esterase with advanced maturity,

[4] The HCl-soluble fraction was not analyzed for methoxyl content since the methoxyl groups are largely lost during the acid extraction.

Photomicrographs of portion of olive fruit, stained with methylene blue. All figures × 109, with same staining and photographic schedule.

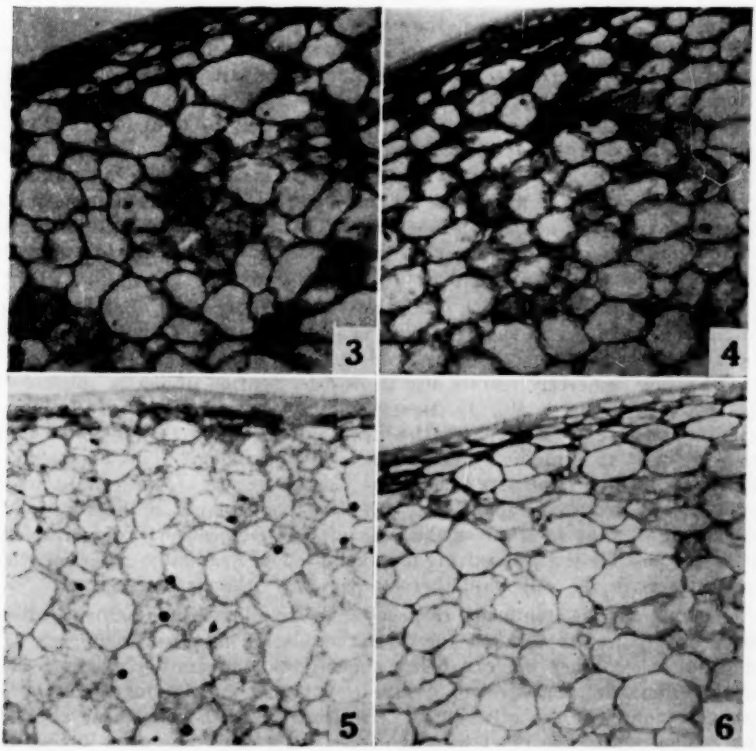

Figure 3. Fresh Sevillano olive. The two densely-staining cells are sclereids. Note heavy stain in cell layers just below surface.

Figure 4. Sevillano olive held 26 weeks in 6% NaCl brine. Changes in staining quality are not detectable.

Figure 5. Fresh Ascolano olive, cooked 5 minutes. Dark spots in flesh are the oil-bearing structures.

Figure 6. Fresh Sevillano olive, after lye treatment and leaching in distilled water.

leading to a decreased methoxyl content. McCready and McComb *(8)* and Eggenberger *(3)* have also presented evidence for this development in various fruits.

A second relationship which can lead to firming involves the osmotically-active solute content. Thus, perhaps by dehydration of the cellulosic, pectic, and other colloidal materials of the cell wall, a toughening of texture may be achieved. Note, for example, the results of tests with varying concentrations of NaCl and sucrose. Likewise, the higher concentrations of $CaCl_2$ may have this sort of influence. (Sufficient calcium ion probably is available in the cell sap for the formation of calcium pectate).

One of the most heuristic questions raised by this investigation concerns the apparent "diminution" of polygalacturonase activity during brining. Although the fruits would soften markedly in the face of such activity on the tree (see *8)*, they remain relatively firm in the brine. [There was no unidirectional change of intrinsic viscosity *(i.e.*, size of the molecule) of the pectic materials with time in the salt solution (Table 6)]. Even in distilled water little softening took place. Several explanations may be advanced for the lack of softening: (a) in the light of work with other fruits *(8)*, polygalacturonase probably is not available until later stages in maturation; (b) polygalacturonase activity might be inhibited by the composition of the brine or the anaerobic circumstances of brine storage; (c) larger amounts of polyvalent cations in the pectic materials of the cell well could render these materials more resistant to enzymic, as well as thermal (see *13)*, degradation.

The effect of holding time on the texture of the cooked olive is also an intriguing problem. It is known that heating results primarily in a glycosidic breakdown of the pectic materials *(6)*. If the suggestion be accepted that heating indirectly results also in de-esterification of pectic substances (as was shown in potato, *1)*, then possibly the promotion of additional calcium pectate formation by the heat activation of the enzymes may serve in part to explain the firming action (see above). This explanation would necessitate assuming either that pectic esterase becomes more available or that it becomes more active as the olive remains in the brine. (It is known, *6*, that salts affect the rate of de-esterification.) Nevertheless, it is also possible that the salting may bring about gradually closer micellar associations in the cell wall, thereby providing the firmer texture.

During the lye treatment, in processing the olives, the alkaline medium itself is a powerful catalyst for de-methoxylation of pectic materials. In addition, the higher pH can bring about de-polymerization of the pectin chain. The softening effect of lye treatment may well be due to the latter phenomenon. On the other hand, the leaching with tap water supplies polyvalent cations to react with the free acid groups produced during lye treatment and thus undoubtedly contributes the firming effect found in this experiment.

The increase in mechanical strength with immersion of olives in 2.5% NaCl filling solution probably is also due to dehydration. When calcium ions are added to this filling solution, they can react with whatever free acid groups may still be available after the leaching. However, the retorting operation leads to further softening, presumably as a result of additional depolymerization induced by the heat.

SUMMARY

Mechanical textural tests and chemical analyses were made of two varieties of olives under various conditions of holding brine storage. The strength of the cooked or canned fruit tissues increased progressively with time of storage. This increase in firmness can be related to increasing de-esterification (plus calcium neutralization of the newly-formed free carboxyl groups) of the pectic materials of the cell walls. Likewise it may be related to the dehydration role of the solutes in the holding brine.

TABLE 6

Changes in pectic material of 1953 olives during brine storage[1]

Holding time (weeks)	Total calcium (% of anhydrogalacturonic acid) (all fractions)			Methoxyl content (% of anhydrogalacturonic acid) (Versene-soluble fraction)			Intrinsic viscosity (Versene-soluble fraction)		
	Firm-green	Soft-greenish-yellow	All	Firm-green	Soft-greenish-yellow	All	Firm-green	Soft-greenish-yellow	All
0	1.70	1.60	1.65	4.75	7.21	5.98	2.0	2.6	2.3
2	2.24	2.29	2.27	1.90	3.05	2.67	1.5	4.4	3.2
4	1.99	3.15	2.57	3.38	2.61	2.99	2.2	2.9	2.5
8	1.72	3.04	2.38	3.49	2.16	2.82	2.8	3.0	2.9
12	2.58	.....[2]	2.58	4.40	.....[2]	4.40	2.2	....[2]	2.2

[1] Averages for both varieties.
[2] Insufficient material for analysis.

Further de-esterification and depolymerization of pectic substances appear to be the consequence of the lye treatment during the dark-ripe process. Subsequent firming may be provided in later stages of processing where calcium or other polyvalent cations are present in ambient solutions.

LITERATURE CITED

1. Bettelheim, F. A., and Sterling, C. Factors associated with potato texture. II. Pectic substances. *Food Research*, 20, 118-129 (1955).
2. Cruess, W. V. *Commercial Fruit and Vegetable Products* 3rd Ed. 1948, McGraw-Hill Book Co., Inc., New York.
3. Eggenberger, W. Biochemische Untersuchungen an Äpfeln während der Entwicklung und Lagerung. *Ber. Schweiz. Bot. Ges.*, 59, 91-154 (1949).
4. Fabian, F. W., and Johnson, E. A. Experimental work on cucumber fermentation. Parts IX, X, XI, and XII. Mich. Agr. Exp. Sta. *Tech. Bull.*, 157 (1938).
5. Johansen, D. A. *Plant Microtechnique*. 1940, McGraw-Hill Book Co., Inc., New York.
6. Kertesz, Z. I. *The Pectic Substances*. 1951, Interscience Publ., Inc., New York.
7. McCready, R. M., and McComb, E. A. Extraction and determination of total pectic materials in fruits. *Anal. Chem.*, 24, 1986-1988 (1952).
8. McCready, R. M., and McComb, E. A. Pectic constituents in ripe and unripe fruit. *Food Research*, 19, 530-535 (1954).
9. McCready, R. M., and McComb, E. A. Texture changes in brined cherries. *Western Canner & Packer*, 46 (13), 17, 19 (Dec., 1954).
10. McCready, R. M., and Reeve, R. M. Test for pectin based on reaction of hydroxamic acids with ferric ion. *J. Agr. Food Chem.*, 3, 260-262 (1955).
11. Nortje, B. K., and Vaughn, R. H. The pectolytic activity of species of the genus *Bacillus:* Qualitative studies with *Bacillus subtilis* and *Bacillus pumilis* in relation to the softening of olives and pickles. *Food Research*, 18, 57-69 (1953).
12. Owens, H. S., McCready, R. M., Shepherd, A., Schultz, T. H., Pippen, E. L., Swenson, H. A., Miers, J. C., Erlandsen, R. F., and Maclay, W. D. Methods used at Western Regional Research Laboratory for extraction and analysis of pectic materials. U. S. Department of Agriculture, Western Reg. Res. Lab., Albany, Calif. A1C-340 (1952).
13. Reeve, R. M., and Leinbach, L. R. Histological investigations of texture in apples. I. Composition and influence of heat on structure. *Food Research*, 18, 592-603 (1953).
14. Sterling, C. Sclereid development and the texture of Bartlett pears. *Food Research*, 19, 433-443 (1954).
15. Vaughn, R. H., Levinson, J. H., Nagel, C. W., and Krumperman, P. H. Sources and types of aerobic microorganisms associated with the softening of fermenting cucumbers. *Food Research*, 19, 494-502 (1954).

SIMULTANEOUS VS. SUCCESSIVE PRESENTATION IN A PAIRED COMPARISON SITUATION[a]

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Laboratory and home panels often share similar objectives; yet the differences between these approaches extend beyond population size and representativeness of sample. Advocates of the laboratory approach can make an excellent case for rigidly controlled conditions, attentive observers, and freedom from incidental distractions. Market researchers, on the other hand, find greater comfort in larger, more representative groups and in testing under actual home conditions. The differences between these methods are well recognized, and so long as they do not collaborate on a particular problem the issue does not threaten either researcher. It is only in situations where both methods have been applied, with apparently conflicting results, that the adversaries will find it necessary to justify their results. Marked preferences have left the Psychometrics Laboratory of the United States Testing Company for the field and returned with a 50-50 split and surprisingly little consumer interest in what had been regarded as a highly improved formulation. Conversely, field preferences have not withstood the rigors of laboratory preparation and evaluation. For some of these differences in results, either side may have a ready answer, but to explain others some research into the two methods may be necessitated.

Preference testing in the laboratory, as is well known, may consist of paired comparisons, absolute ratings, rankings, or combinations of all three. Preference testing in the field most frequently can be described as successive presentations in which the housewife is requested to prepare sample A first, and sample B a few days later. In addition to the serving at meal times, the samples in the field study are also being examined successively, a situation far more representative of typical consumer behavior.

The present study was designed to measure the nature and extent of the differences between the two approaches; the interval between samples was selected as the primary variable. An attempt was made to duplicate in the laboratory one aspect of the home test situation and to compare the findings with those of an orthodox laboratory evaluation.

METHOD

In brief, the design of the experiment was to conduct a series of paired comparison preference tests on two pairs of soups. For each pair of samples, five such tests were performed: one was under conditions of simultaneous presentation of samples, and the remaining four involved an interval between samples of 1, 3, 7, and 10 days.

The samples tested were two pairs of canned soups. Before the start of the experiment itself, a large number of soups were subjected to flavor preference testing in order

[a] Presented at the 15th Annual Meeting of the Institute of Food Technologists, June 15, 1955, Columbus, Ohio.

to select experimental samples covering a range of preferences. Finally, two pairs of soups were selected; the first pair was composed of two onion soups which had been found to differ widely in consumer acceptance. The second pair consisted of two pea soups; here, there was only a moderate preference for one sample over the other. In this report, those onion and pea soup samples which performed best in preliminary testing will be referred to as the "preferred" soups, and the other two samples as the "non-preferred" soups.

Two panels of consumers were utilized; one panel was used for all the tests involving the onion soups and the other was used for the pea soups. Each panel was composed of 60 men and women. All panel members were experienced in flavor preference testing, but were unaware of the purpose of the experiment.

The flavor testing took place in the Psychometrics Laboratory of United States Testing Company. Flavor evaluations were made in our multiple testing station, wherein independent judgments are assured. The soup samples were served in coded cups, and variables such as serving temperature, color and quantity were carefully controlled.

After having tasted both soups of a pair, the panel member recorded his judgment on the form shown in Figure 1. This form requires a report of the direction of preference

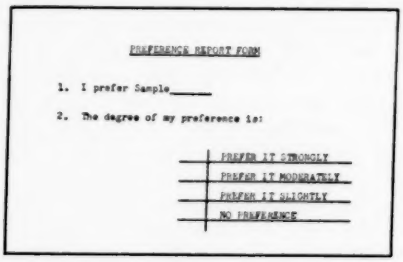

Figure 1. Form used by consumers for reporting direction and degree of preference.

and, secondly, of the degree of that preference. The same form was used for all preference judgments.

According to the method of analysis developed by Scheffé *(2)*, each judgment can be transposed to the scale shown in Figure 2. This combines the direction and degree of preference into a single linear scale. For example, a "strong" preference for Sample X would be assigned a value of +3 on this scale, a report of "no preference" would be zero, and a "moderate" preference for Sample Y would be −2. In a single preference test 60 such ratings were obtained, and the mean of these ratings was taken as a measure of the preference for one sample over the other.

Each pair of soups was subjected to a series of five paired-comparison preference tests over a period of several months. The first of these tests was a simultaneous paired

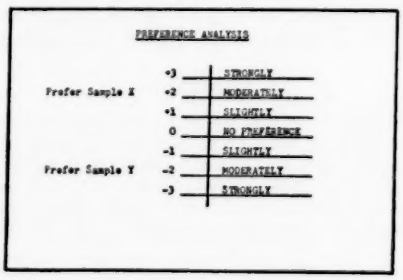

Figure 2. Analysis of consumer preference ratings.

comparison in which the panel members were presented with the two soups at the same time, and preference judgments were reported after tasting back and forth between the two samples. The other 4 tests were successively paired comparisons, in which the 2 samples were separated by an interval of 1, 3, 7, and 10 days. Here, the panel members were presented with a single sample, let us say onion soup A, and asked to remember it for future evaluation. After the appropriate interval, they were presented with onion soup B and asked to record their preference between the two soups. Several weeks later, each successive paired comparison was repeated with the order of sample presentation reversed. Thus, totalling the results for the two sessions would yield a preference with order of presentation balanced out.

RESULTS

The method of scoring described was applied to the data obtained in the preference tests and the resulting mean preferences are shown in Table 1 and Figure 3. In Figure 3,

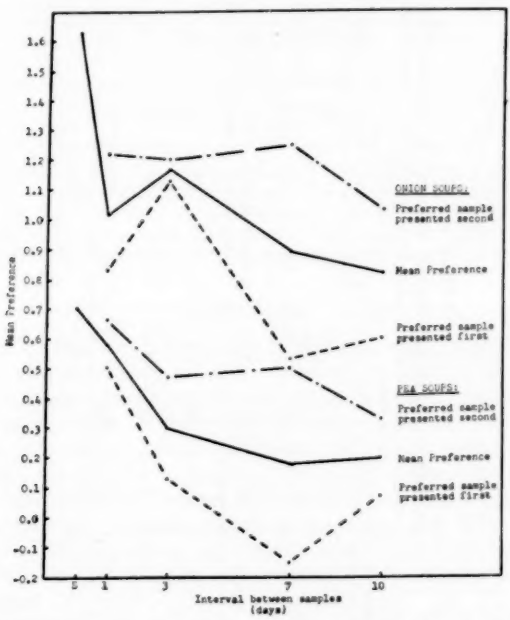

Figure 3. Mean preference ratings as a function of the length of the interval between samples.

the mean preferences are plotted against the length of the interval between samples. The data obtained on the onion soups, where there was a strong preference, are shown at the top of the graph. For example, the highest point in the upper left hand corner represents the results obtained in the simultaneous paired comparison between the two onion soups. Here, a preference rating of +1.6 was obtained. This rating is based upon a preference split of 50 to 4 in favor of the preferred soup, with 6 no preference judgments. Thus it can be seen that a mean rating as high as +1.6 on this scale represents a very strong preference for one sample over the other.

The two solid lines in Figure 3 show the preference ratings obtained when the results are analyzed according to the typical balanced order of presentation; that is, when each sample has been presented first with equal frequency. Here we see that the

TABLE 1

Obtained preference distributions and mean preference ratings

Onion soups	Simultaneous presentation	1 day apart			3 days apart			7 days apart			10 days apart		
		P-NP	NP-P	Total	P-NP	NP-P	Total	P-NP	NP-P	Total	P-NP	NP-P	Total
Number preferring "P"[1]	50	39	43	82	41	44	85	36	42	78	32	40	72
Number preferring "NP"[2]	4	13	5	18	9	7	16	17	6	23	12	11	23
No preference	6	8	12	20	10	9	19	7	12	19	16	9	25
Mean rating	1.63	.83	1.22	1.02	1.13	1.20	1.17	.53	1.25	.89	.60	1.03	.82
Pea soups													
Number preferring "P"	39	35	36	71	31	33	64	26	35	61	22	29	51
Number preferring "NP"	20	19	15	34	26	18	44	32	19	51	17	22	39
No preference	1	6	9	15	3	9	12	2	6	8	21	9	30
Mean rating	.70	.50	.66	.58	.13	.47	.30	−.15	.50	.18	.07	.33	.20

[1] "P" = Preferred sample.

[2] "NP" = Non-preferred sample.

strongest preferences, with both sets of samples, were obtained under conditions of simultaneous presentation. Furthermore, as the interval between samples is lengthened, there appears to be a tendency for the preference to diminish. For example, the degree of preference for the preferred pea soup declines from +.70 under simultaneous presentation to +.20 when a 10-day period intervenes between samples.

This decrease in preference occurs in both sets of samples but is more pronounced where a higher preference exists originally.

The influence of order of presentation upon preference is represented by the broken lines on the graph. Here it appears that a striking position effect has occurred. The top line in each set of three represents the results obtained when the preferred onion or pea soup was presented *second*. In every case, the mean preference under these conditions was higher than when the preferred sample was presented first. The relative acceptance of the two soups, then, is clearly dependent upon their order of presentation, and in this study it appears that the second sample is consistently preferred.

It will be recalled that the primary purpose of this study was to measure the influence of the length of the interval between samples. An examination of Figure 3 reveals the existence of a relationship between this variable and position effect. The position effect appears to be relatively weak when the inter-sample interval is as short as 1 or 3 days; that is, the results obtained are fairly similar, whichever soup is presented first. With an interval between samples of 7 days, the position effect reaches its greatest magnitude. In the case of the pea soups, the combined influence of a 7-day interval between samples and a non-balanced order of presentation is so great as to result in a negative preference rating, that is, an apparent preference slightly in favor of the non-preferred soup.

The obtained finding that the sample presented second achieves a relatively higher preference appears at first glance to contrast with the results obtained by Hanson *et al. (1)*. In their study, involving successive absolute ratings, the first sample judged tended to receive a higher rating than succeeding samples. This apparent conflict, however, can perhaps be attributed to the widely differing methods employed in the two studies.

DISCUSSION

The results of this exploratory study have shown considerable differences between the simultaneous and successive paired comparisons. In summary, the simultaneous presentation yields stronger preferences than does the successive. In addition, the successive paired comparison is vulnerable to the influence of order of presentation, and becomes more so as the interval between samples increases. This phenomenon may be a partial contributor to the lesser preferences obtained with successive presentation, since any non-relevant variable which is operative, yet balanced out, is bound to have a levelling effect upon preference. With simultaneous comparison, on the other hand, order of presentation is eliminated as a contributing variable.

The implications of these findings extend from the small laboratory panels to the large panels employed in home testing situations. It is often difficult to reconcile the marked preferences obtained in laboratory situations with the often non-differentiated field results. The present study indicates that one explanation for such differences may be found in the fact that home preference testing is most frequently successive, whereas laboratory testing tends to lean more heavily upon simultaneous presentations. At this point it would be unreasonable to assume a partisan attitude toward either approach to measurement. It is difficult and costly for a research group to gather parellel data from the two sources, and inter-laboratory effort is the most efficient approach to the solution of this problem.

LITERATURE CITED

1. HANSON, H., DAVIS, J., CAMPBELL, A., ANDERSON, J., AND LINEWEAVER, H. Sensory test methods II. Effect of previous tests on consumer response to foods. *Food Technol.*, **9**, 56 (1955).
2. SCHEFFÉ, H. An analysis of variance for paired comparisons. *J. Am. Stat. Assn.*, **47**, 381 (1952).

THE HYDROXYPROLINE CONTENT OF ANIMAL TISSUES[a]

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Values for the amount of eighteen different amino acids in animal tissues determined by microbiological assay have been reported *(2, 5, 6, 10, 11, 13)*. The nitrogen present in these amino acids accounts for 87% of the total nitrogen in the tissue. The remainder of the nitrogen can be partially accounted for as vitamins, purines and pyrimidines, other nitrogen-containing metabolites and amide nitrogen, but the presence of small quantities of some of the less common amino acids is possible. Alexander *(1)* identified hydroxyproline in hydrolysates of beef round and pork ham by using paper chromatographic methods and by the technique of Moore and Stein with an ion-exchange column estimated the amount to be 0.7 g./16 g. N and 0.6 g./16 g. N., respectively. This work indicated that the quantity of hydroxyproline was sufficient to warrant quantitative determination in several animal tissues. A suitable microbiological procedure for the determination of hydroxyproline could not be found in the literature and evidence had been reported by Dunn *et al. (4)* that discouraged any attempt at devising one. Several colorimetric methods were available *(8, 9, 12)*, however, and a modification of that of Troll and Cannan *(12)* was used. This method is based on the reaction of hydroxyproline and proline with ninhydrin in phosphate buffer (pH 7.0) to yield a red pigment which can be extracted with benzene. Hydroxyproline is determined quantitatively by making a comparison of the absorption at 570 mμ, the maximum for hydroxyproline, and 550 mμ, the maximum for proline.

MATERIALS AND METHODS

The animal tissues employed for these determinations were the lyophilized, fat-extracted samples described by Violante *et al. (13)*. The samples were hydrolyzed with 5 N KOH (25 ml./g. of sample) in metal beakers at 121°C. and 15 lbs. pressure for 5 hours, the maximum release time. The alkaline hydrolysates were placed in the refrigerator overnight, then neutralized with 10 N HCl, filtered and made up to volume with 0.1 M phosphate buffer. It was found that acid-hydrolyzed samples could not be used because the ninhydrin would react with the humin that was formed to yield a brown-colored compound which was benzene soluble.

For convenience the determinations were run in groups of three or four. One-tenth ml. of phosphate buffer and 0.1 ml. of sample were pipetted into 10-ml. volumetric flasks, 2.5 ml. of benzene added and the flasks placed in a steam-heated water bath at 75°C. Two-tenths ml. of ninhydrin solution in methoxy Cellosolve (2-methoxy ethanol) were added and the flasks were shaken unstoppered for 5 minutes by means of a rapid vibrator arranged in the water bath. Evaporation was limited by the constricted neck of the flasks and further controlled by immersing them only to the level of the benzene. The flasks were removed, cooled and diluted to volume with benzene directly in the reaction flasks. The benzene layer was decanted through glass wool directly into quartz cuvettes and optical density determinations were made in a Beckman Spectro-

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photometer at 570 mμ and 550 mμ. The correction equation of Troll and Cannan *(12)* was applied. By using hydroxyproline whose purity had been checked by paper chromatography and optical rotation we could only obtain 80% of the theoretical color yield for hydroxyproline as calculated from the millimolar extinction coefficient. To correct for this low color yield a standard containing 1 μmole hydroxyproline/ml. was run with each group and the sample results were based on the value for the standard. Color yield for each sample was shown to depend upon ninhydrin concentration and upon benzene volume used during the reaction. This relation could best be expressed by a ratio of ninhydrin concentration to benzene volume. This ratio gave an expression for effective concentration of ninhydrin at the benzene-phosphate buffer interface. The ratio that gave maximum color yield for each sample was determined and used. Maximum value obtained for most samples was *at a ratio of* 0.12, i.e., 0.3 M ninhydrin in methoxy Cellosolve, 2.5 ml. of benzene. By this method 98% recovery of hydroxyproline was obtained from a proline-hydroxyproline mixture and 90% recovery of hydroxyproline was obtained from a mixture of beef tongue, hydroxyproline, and proline (Table 1).

RESULTS

The hydroxyproline contents of 8 muscle tissues are very similar. However, there is a marked increase of hydroxyproline in beef tongue, kidney and heart. Beef liver contains about the same amount as muscle tissue and beef brain contains somewhat less. Results are summarized in Table 2. Hydroxyproline could be due to collagen not removed during sample preparation. Hydroxyproline content of collagen or gelatin is approximately

TABLE 1

Recovery of crystalline hydroxy-*l*-proline

Sample	Hydroxyproline found, mg.	Recovery, %
1) 5.8 mg. hydroxyproline 18.6 mg. proline	5.8	100
2) 5.8 mg. hydroxyproline 18.6 mg. proline 90.0 mg. lyophilized beef tongue	7.9	89.7
3) 90.0 mg. lyophilized beef tongue	2.7	....

TABLE 2

Hydroxyproline content of animal tissues

Meat cut	g./16 g. of N	g./100 g. free-tissue
Beef round	0.8	0.17
Beef shoulder	0.8	0.17
Veal round	0.9	0.20
Veal shoulder	0.9	0.18
Pork ham	0.9	0.16
Pork shoulder	0.9	0.14
Lamb leg	0.7	0.12
Lamb shoulder	0.9	0.16
Beef tongue	1.7	0.28
Beef heart	1.1	0.18
Beef liver	0.9	0.16
Beef kidney	1.2	0.20
Beef brain	0.5	0.06

13% *(3, 12)*. If it is assumed that muscle protein does not contain hydroxyproline, as reported by Lang *(7)*, our work indicates there is approximately 1.0 g. of collagen per 100 g. of fresh muscle tissue. Since collagen is also abnormally high in glycine, containing approximately 23% *(3)*, a check is possible by comparing glycine contents of various tissues. This is shown in Table 3. Glycine *(2)* is seen to parallel hydroxyproline sufficiently close to indicate that hydroxyproline is due to tissue collagen. Collagen content would be subject to biological variation between animals.

TABLE 3

Comparison of hydroxyproline and glycine in tissues

Meat cut	Hydroxyproline as g./16 g. N	Glycine[1]
Muscle meats	0.8±0.05	4.5±0.15
Beef tongue	1.7	6.5
Beef kidney	1.2	6.0
Beef heart	1.1	4.7
Beef brain	0.5	4.2

[1] From *J. Nutrition*, **51**, 319 (1953).

SUMMARY

The application of a colorimetric method to the determination of hydroxyproline in various animal tissues is reported. Muscle tissues from 4 species (beef, veal, pork, lamb) were shown to contain approximately 0.9 g. of hydroxyproline per 16 g. of N. The relation of the hydroxyproline to tissue collagen is discussed.

LITERATURE CITED

1. Alexander, J. C. (University of Wisconsin, Ph.D. Thesis, 1954.)
2. Alexander, J. C., Beckner, C. W., and Elvehjem, C. A. The alanine, cystine, glycine and serine content of meat. *J. Nutrition*, **51**, 319 (1953).
3. Block, R. J., and Bolling, D., in *The Amino Acid Composition of Proteins and Foods*, 1951, Charles C. Thomas, Springfield, p. 486.
4. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B. The amino acid requirements of *Leuconostoc mesenteroides* P-60. *J. Biol. Chem.* **156**, 703 (1944).
5. Greenhut, I. T., Potter, R. L., and Elvehjem, C. A. The phenylalanine and tryptophan content of meat. *Arch. Biochem.*, **6**, 177 (1945).
6. Greenhut, I. T., Sirny, R. J., and Elvehjem, C. A. The lysine, methionine and threonine content of meats. *J. Nutrition*, 35, 689 (1948).
7. Lang, K. Eine Mikromethode zur bestimmumg des Prolins und des Oxyprolins. *Z. für Physiol. Chem.*, **219**, 148 (1933).
8. Neuman, R. E., and Logan, M. A. The determination of hydroxyproline. *J. Biol. Chem.*, **184**, 299 (1950).
9. Rogers, C. J., Kimmel, J. R., Hutchin, M. E., and Harper, H. A. A hydroxyproline method of analysis for a modified gelatin in plasma and urine. *Ibid.*, **206**, 553 (1954).
10. Schweigert, B. S., Tatman, I. E., and Elvehjem, C. A. The leucine, valine, and isoleucine content of meat. *Arch. Biochem.*, **6**, 177 (1945).
11. Sirny, R. J., Greenhut, I. T., and Elvehjem, C. A. The arginine and histidine content of meat. *J. Nutrition*, **41**, 383 (1950).
12. Troll, W., and Cannan, R. K. A modified photometric ninhydrin method for the analysis of amino and imino acids. *J. Biol. Chem.*, **200**, 803 (1953).
13. Violante, A. M., Sirny, R. J., and Elvehjem, C. A. The aspartic acid, glutamic acid, proline and tyrosine content of meat. *J. Nutrition*, **47**, 307 (1952).

STUDIES ON HEAT RESISTANCE. VII. THE EFFECT OF PHOSPHATE ON THE APPARENT HEAT RESISTANCE OF SPORES OF *BACILLUS STEAROTHERMOPHILUS*[a]

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(Manuscript received August 18, 1955)

The necessity of having accurate values for the spore resistance of organisms significant in food spoilage was recognized at the start of scientific processing studies. A procedure which would yield reproducible heat resistance results was described by Bigelow and Esty *(1)*, and with slight modifications, especially by Esty and Cathcart *(4)* and Esty and C. C. Williams *(5)*, has been used extensively since. A number of other procedures have been described, but since these differ from the Bigelow and Esty method chiefly in the mechanics of the exposure to heat, discussion of them is not pertinent to the present report.

The modification of Esty and C. C. Williams introduced the use of M/15 neutral phosphate buffer as a suspending medium for the spores being heated. No reason was given for the selection of M/15 in preference to some other buffer strength. There is a limited choice of approximately neutral buffer solutions suitable for use in heating experiments, and it seemed probable that the selection of this phosphate concentration was influenced by the publication of Clark *(3)* in which the preparation of Sorenson standards was described. Shortly before his death Dr. Esty agreed that this was probably the determining factor. Regardless of the reason, many determinations of "phosphate resistance" have been made in M/15 buffer, apparently without question of its suitability for use. Townsend, Esty and Baselt *(9)* did note that "two cc. of standard phosphate added to a tube of enrichment medium appears to have a definitely inhibiting effect on the germination of spores" but this observation seems not to have had any influence on subsequent heat resistance studies. Johnson *(7)* in an exploration of this observation by Townsend *et al.* found that phosphate showed concentration effects on both growth and heat resistance of *Bacillus stearothermophilus* more strikingly than with P.A. 3679 or *Clostridium thermosaccharolyticum*, although growth from spores of these latter two organisms was adversely affected by concentrations of phosphate ranging from M/10 for P.A. 3679 to M/30 to M/50 for *Cl. thermosaccharolyticum*.

These observations by Townsend *et al.* and Johnson appeared to be of sufficient interest for studies of heat resistance and spore germination of the flat sour type to justify extension. Accordingly, the work here reported was undertaken to investigate especially the effect of the concentration of phosphate in the suspending medium on the spore resistance of several strains of this organism.

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[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned Number 590 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

MATERIALS AND METHODS

The organisms used were originally isolated from underprocessed canned food. They were obtained through the kindness of C. W. Bohrer and C. C. Williams of the Research Laboratory of the National Canners Association. Two facultatively and 9 obligately thermophilic strains, according to the scheme of Cameron and Esty *(2)*, were included.

The percentage of sporulation after 2 weeks at 55°C.(131°F.) was compared for two media-standard nutrient agar with 0.1% soluble starch, and a basamin medium which had been found in other studies to be an especially good sporulation medium. The formula for this medium is:

Basamin (Busch)	0.3%
Glucose	0.2%
Na_2HPO_4	0.25%
KH_2PO_4	0.1%
NaCl	0.1%
NH_4Cl	0.1%
*Mineral solution	0.1%
Agar	2.0%
pH 7.2	

* Mineral solution: $MgSO_4$—0.5 g., $FeSO_4$—0.5 g., $CaCl_2$—0.5 g., distilled water—100 ml.

One strain (1518) sporulated slightly better on the starch-nutrient agar than on the basamin medium. All other spores were grown on the basamin medium.

Spores were harvested from Roux bottle cultures with distilled water, and washed 3 times by centrifugation with distilled water. They were suspended in concentrated suspensions in distilled water, filtered through a gauze and cotton filter to remove clumps, pasteurized at 85°C.(185°F.) for 15 minutes, and standardized by dilution plating. The stock suspensions were stored at 5°C.(41°F.).

Johnson noted that potassium phosphate appeared to add some toxicity in excess of that noted with all sodium phosphate buffer in the instance of the more concentrated buffers. Consequently, the buffers used in the present experimentation were made with mixtures of mono- and disodium phosphates. The concentrated spore suspensions were diluted to give 10,000 spores per ml. with each of the buffer strengths and with distilled water, each distributed in aliquots of 2 ml. to pyrex tubes, sealed and heated in groups of 5 at 120°C.(248°F.) exactly according to the Esty and Williams procedure.

The recovery medium was a brain-heart infusion prepared from fresh tissues. The superiority of the fresh medium was established by comparing its productivity for each strain with that of a similar medium prepared from a dehydrated base. The medium was prepared by infusing 200 g. of calf brains and 250 g. of beef heart for 1½ hours in 500 ml. of water. The infusion was clarified and enriched with proteose peptone—10 g., glucose—2 g., NaCl—5 g., Na_2HPO_4—2.5 g. and soluble starch—1 g. The volume was made up to one liter, the pH adjusted to 7.4 and the medium distributed in 5-ml. quantities and sterilized in the usual way.

Rough determinations of resistance were made by sampling at 10-minute intervals. The final runs were sampled at 2- or 4-minute intervals. Since dormancy has never been considered to be a problem with the flat sour group, final observations of the recovery cultures were made on the 5th day of incubation. The accuracy of the values given has been established by several replications.

RESULTS

The data recorded for comparative heating experiments with spores suspended in distilled water and in phosphate buffers of M/15, M/30, M/60 and M/120 strengths are shown in Table 1.

Although there are a few minor irregularities among the data, the general trend of the results clearly suggests that a more dilute buffer than M/15 should be used in determining phosphate resistance of strains of *B. stearothermophilus*. The results speak for the use of a buffer not stronger than M/60.

In order to determine whether the results observed probably were due to interference by phosphate with the germination of heated spores, or to real differences in

survival times with the different buffers, a series of determinations was made in which the spores were heated in different levels of phosphate, and subcultured into medium adjusted with added phosphate so that the final phosphate concentration of each tube after the addition of the inoculum was equal, and was the same as that which resulted when 2 ml. of M/15 buffer carried the inoculum.

The results of the determinations for 3 strains are shown in Table 2.

It is obvious that the phosphate concentration obtained by adding 2 ml. of M/15 buffer to 5 ml. of medium is inhibitory to the germination of heated spores. Whether these spores which have the potentiality of growth, as is shown by their germination in media with lower phosphate concentrations, can overcome the inhibitory effects of the more concentrated phosphate is not known, but is certainly a possibility.

DISCUSSION

Replicate determinations of spore resistance to heat may agree under a given set of conditions, but vary considerably with relatively minor changes in the conditions of exposure or recovery. Thus it is well established that the endpoints observed can be influenced considerably by some

TABLE 1

Thermal death time determinations at 120°C. (248°F.) of obligate and facultative thermophiles in disodium phosphate buffer

	Molar concentration of buffer									
	M/15 minutes		M/30 minutes		M/60 minutes		M/120 minutes		Dist. H_2O minutes	
	+	−	+	−	+	−	+	−	+	−
Facultative strains										
1518	26	28	34	36	56	60	60	64	52	60
10253	6	8	12	14	16	18	16	18	12	14
Obligate strains										
11330	32	34	48	50	44	48	60	64	52	54
5540	8	12	18	20	24	26	30	32	26	30
7595	30	32	40	44	50	54	56	60	50	54
5732	36	40	40	44	48	52	56	60	48	52
2263	2	4	2	4	8	10	8	10	4	6
10266	14	16	18	20	18	20	24	26	14	16
2156	10	12	14	16	14	16	16	18	12	14
3656	2	4	8	10	10	12	12	16	8	10
3441	8	10	10	12	10	12	18	20	12	14

+ = growth, − = no growth.

TABLE 2

Effect of supplemental phosphate in the recovery medium on the germination of heated spores of Bacillus stearothermophilus

PO_4 molality of buffer in which heated	Minutes survival at 120°C. (248°F.)					
	Strain					
	3656		10266		1518	
	No extra PO_4	Added PO_4	No extra PO_4	Added PO_4	No extra PO_4	Added PO_4
0	8	4	12	10	48	32
M/15[1]	2	2	8	8	26	28
M/30	8	2	12	8	32	28
M/60	10	2	12	10	56	32
M/120	12	4	14	10	56	32

[1] No supplement added.

one or another of several environmental factors, such as the pH of the suspending fluid, the temperature of incubation of the recovery culture, or the composition of the recovery medium. Sorenson's phosphate buffer has long been used in order to avoid the small, but important, changes in pH which sometimes may be experienced with unbuffered suspending medium. The results here presented indicate that many of the data which have been recorded for the spore resistance of flat sour bacteria may not reflect the true "phosphate resistance." Heated spores of these organisms seem to be rather sensitive to the concentration of phosphate in the recovery medium, and the Sorenson mixture may introduce more phosphate than is desirable.

In the practical use of heat resistance data in calculating process times for a commodity it is ordinarily considered important that the maximum values for the organism of most significance in the product be the ones used. It would seem likely that some, possibly many, of the determinations made with flat sour spores do not truly reflect the maximum which could be obtained. However, any error which might possibly be created through the use of apparent rather than true resistance is of minor or no practical importance. Experience speaks for the general adequacy of the heating times which have been calculated and used. In some cases this is probably due to the fact that the spore resistance of the experimental strain (1518) is greater than the resistance of most spores encountered in nature. Thus there is a margin of safety inherent in the data used for calculations which is in excess of any possible error due to the use of a low end point. Furthermore, with a large volume of recovery medium in relation to the amount of buffer spore suspension used for exposure the phosphate concentration could be diluted to a beneficial level. It is apparent from the data of each table that a low level of phosphate in the recovery medium favors obtaining high resistance values. This is not surprising since Fred and Hart *(6)* and Mallmann and Gallo *(8)* have each noted a stimulating effect of phosphate on bacterial activity.

Although delayed germination has never been thought to be a factor with flat sour spores the fact that potentially viable spores may be present in recovery cultures containing an inhibitory level of phosphate suggests that it is a possibility.

SUMMARY

Spores of *Bacillus stearothermophilus* heated in disodium phosphate buffer show an apparent increase in resistance with decreasing molal concentration of phosphate over the range M/15 to M/120. Although there is possibly some real difference in resistance attributable to the molality of the buffer in which heated, most of the differences recorded appear to be due to an inhibitory effect of the phosphate added by the buffer to the recovery medium in the concentrations greater than M/120. There seems to be a stimulation of germination and growth, as shown by an apparent greater resistance, by the M/120 buffer as compared with distilled water and greater phosphate concentrations.

LITERATURE CITED

1. Bigelow, W. D., and Esty, J. R. The thermal death point in relation to time of typical thermophilic organisms. *J. Inf. Dis.*, 27, 602–617 (1920).

2. CAMERON, E. J., AND ESTY, J. R. The examination of spoiled canned foods. 2. Classification of flat sour types. *J. Inf. Dis.*, **39**, 89–105 (1926).
3. CLARK, W. M. *The Determination of Hydrogen Ions.* 1920, Williams and Wilkins, Baltimore, Md.
4. ESTY, J. R., AND CATHCART, P. H. The change in the hydrogen ion concentration of various mediums during heating in soft and pyrex glass tubes. *J. Inf. Dis.*, **29**, 29–39 (1921).
5. ESTY, J. R., AND WILLIAMS, C. C. Heat resistance studies. I. A new method for the determination of heat resistance of bacterial spores. *J. Inf. Dis.*, **34**, 516–528 (1924).
6. FRED, E. B., AND HART, E. B. The comparative effect of phosphates and sulfates on soil bacteria. Wisconsin Agr. Exp. Sta. *Research Bull.* **35**, 35–66 (1915).
7. JOHNSON, D. E. The effect of phosphate on the germination of heated and unheated bacterial spores. (Thesis for the M.A. degree, University of Texas, Austin, 1952.)
8. MALLMANN, W. L., AND GALLO, F. The influence of phosphates on the metabolism of bacteria. *Papers of the Mich. Acad. of Sci., Arts and Letters*, **14**, 617–640 (1930).
9. TOWNSEND, C. T., ESTY, J. R., AND BASELT, F. C. Heat resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. *Food Research*, **3**, 323–346 (1938).

ONE-TAILED AND TWO-TAILED TESTS IN ORGANOLEPTIC COMPARISONS

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Procedures and the basic statistical principles underlying the paired-sample and triangular techniques in organoleptic testing have been described in numerous papers *(2, 3, 4, 5, 6, 8, 11)*. These tests are frequently used (a) to determine whether an experimental product differs from a standard in respect to a given character or to select members of a taste panel capable of distinguishing between a product and a standard, and (b) to compare the quality or preference between two products.

It is the purpose of this paper to supply tables for paired-sample and triangular tests and to discuss briefly which tables are appropriate for each of the cases (a) and (b).

Under case (a) above let us consider the selection of a panel of tasters by means of paired samples. To test the acuity of the group, each taster is given one sample of unsweetened tomato juice and a second sample of the same juice to which a small amount of sucrose has been added. We are interested in the ability of the taster to be able to select "correctly" in a series of trials the sample having the greater sweetness. If he is able to detect this sample with a frequency which will sufficiently *exceed* that to be expected by chance, then we shall infer that this taster does possess some ability in detecting sugar in tomato juice, provided, of course, that our experimental controls are adequate. If this taster were to select the sweet sample "correctly" with a frequency sufficiently below that expected by chance, then we could not infer that he can detect the sweet sample. The result, however, is just as unusual or improbable an outcome as a frequency of the same excess above the chance expectancy. In this case we are interested only in the probability of the frequency of correct choices *exceeding* a given value, and this is properly a one-sided or one-tailed test. A significantly small number of correct choices would have no positive bearing upon the taster's ability to detect sugar. All frequencies of choices less than the significant number of correct choices would constitute negative evidence concerning his ability. These samples could equally well have been presented in a triangular test.

In the second case (b), suppose that the tasters of a panel are given several samples each of which contains two commodities, presented as a paired-sample or triangular comparison, and are asked to indicate which of the two they prefer. If we assume that some will prefer one and some the other in about equal numbers, then a large number of selections of the one commodity over the other may indicate, on the basis of the panel's judgment, a significant quality or preference difference between the two. Since either sample may be the preferred one, the appropriate test is two-sided or two-tailed. The selection of a particular commodity an unusually large number of times is just as interesting as its selection an unusually small number of times.

A limited table for use in determining significance for paired-sample tests in the two cases and a table for use in difference testing in the triangular system have already appeared elsewhere *(1, 9)*. Tables 1 and 2 of this paper, based on cumulative terms of the binomial distribution *(7, 10)*, indicate the number of agreeing judgments (two-tail) or correct answers (one-tail) required for significance in the paired-sample ($p = 1/2$) and triangular ($p = 1/6$) systems, where p is the probability of a correct guess. There has been an expressed desire for such tables by technicians actively engaged in organoleptic research.

For values of n (number of tasters or tastings) not in the tables, formulas based on the corrected normal-curve approximations to the binomial distribution may be used for determining required numbers of agreeing judgments for significance. Similar formulas for the case in which $p = 1/3$ and the method of derivation have been given by Roessler, Warren, and Guymon *(9)*. For n tasters or tastings and $p = 1/6$ the observed number of agreeing judgments (two-tail) or correct answers (one-tail) must exceed

$$\frac{n}{6} + 0.5 + \frac{z\sqrt{5n}}{6}$$

where for the two-tailed test at the 5%, 1%, and 0.1% levels of significance the values of z are, respectively, 1.960, 2.576, and 3.291 and for the one-tailed test the values of z are, respectively, 1.645, 2.326, and 3.090. For the paired-sample comparisons ($p = 1/2$) the corresponding numbers of agreeing judgments or correct answers must exceed

$$\frac{n}{2} + 0.5 + \frac{z\sqrt{n}}{2}$$

where z has the same values as before.

Harrison and Elder *(5)* have supplied charts for determining significance in paired-sample comparisons and in odd-sample selection ($p = 1/3$) in the triangular procedure. The headings of their paired-sample chart ($p = 1/2$) are somewhat misleading since the two-tailed probabilities are headed "Difference Judgments" and the one-tailed "Quality Judgments."

Tables for determining significance are easier to use than charts and the results may be evaluated more accurately. In either case, however, care must be exercised in selecting the appropriate one-tailed or two-tailed chart or table.

LITERATURE CITED

1. AMERINE, M. A., AND ROESSLER, E. B. Techniques and problems in the organoleptic examination of wines. *Proc. Amer. Soc. Enol. 1952:* 97–115 (1952).
2. BENGTSSON, K., AND HELM, E. Principles of taste-testing. *Wallerstein Lab. Commun.*, 9, 171 (1946).
3. BOGGS, M. M., AND HANSON, H. L. Analysis of foods by sensory difference tests. *Advances in Food Research*, 2, 219 (1949).
4. DAWSON, E. H., AND DOCHTERMAN, E. F. A comparison of sensory methods of measuring differences in food qualities. *Food Technol.*, 5, 79 (1951).
5. HARRISON, S., AND ELDER, L. W. Some applications of statistics to laboratory taste testing. *Food Technol.*, 4, 434 (1950).
6. LOCKHART, E. E. Binomial systems and organoleptic analysis. *Food Technol.*, 5, 428 (1951).

TABLE 1

Significance in paired taste tests ($p = \frac{1}{2}$)

No. of tasters or tastings	Minimum agreeing judgments necessary to establish significant differentiation (Two-tail test)			Minimum correct answers necessary to establish significant differentiation (One-tail test)		
	Probability level			Probability level		
	.05	.01	.001	.05	.01	.001
5	....	....	....	5	....	....
6	....	....	....	6	....	....
7	7	....	....	7	7	....
8	8	8	....	7	8	....
9	8	9	....	8	9	....
10	9	10	....	9	10	10
11	10	11	11	9	10	11
12	10	11	12	10	11	12
13	11	12	13	10	12	13
14	12	13	14	11	12	13
15	12	13	14	12	13	14
16	13	14	15	12	14	15
17	13	15	16	13	14	16
18	14	15	17	13	15	16
19	15	16	17	14	15	17
20	15	17	18	15	16	18
21	16	17	19	15	17	18
22	17	18	19	16	17	19
23	17	19	20	16	18	20
24	18	19	21	17	19	20
25	18	20	21	18	19	21
26	19	20	22	18	20	22
27	20	21	23	19	20	22
28	20	22	23	19	21	23
29	21	22	24	20	22	24
30	21	23	25	20	22	24
31	22	24	25	21	23	25
32	23	24	26	22	24	26
33	23	25	27	22	24	26
34	24	25	27	23	25	27
35	24	26	28	23	25	27
36	25	27	29	24	26	28
37	25	27	29	24	27	29
38	26	28	30	25	27	29
39	27	28	31	26	28	30
40	27	29	31	26	28	31
41	28	30	32	27	29	31
42	28	30	32	27	29	32
43	29	31	33	28	30	32
44	29	31	34	28	31	33
45	30	32	34	29	31	34
46	31	33	35	30	32	34
47	31	33	36	30	32	35
48	32	34	36	31	33	36
49	32	34	37	31	34	36
50	33	35	37	32	34	37
60	39	41	44	37	40	43
70	44	47	50	43	46	49
80	50	52	56	48	51	55
90	55	58	61	54	57	61
100	61	64	67	59	63	66

TABLE 2
Significance in triangular taste tests ($p = 1/6$)

No. of tasters or tastings	Minimum agreeing judgments necessary to establish significant differentiation (Two-tail test)			Minimum correct answers necessary to establish significant differentiation (One-tail test)		
	Probability level			Probability level		
	.05	.01	.001	.05	.01	.001
5	4	4	5	3	4	5
6	4	5	6	4	4	5
7	4	5	6	4	5	6
8	5	5	6	4	5	6
9	5	6	7	4	5	7
10	5	6	7	5	6	7
11	5	6	8	5	6	7
12	6	7	8	5	6	8
13	6	7	8	5	7	8
14	6	7	9	6	7	8
15	7	8	9	6	7	9
16	7	8	9	6	7	9
17	7	8	10	7	8	9
18	7	9	10	7	8	10
19	8	9	10	7	8	10
20	8	9	11	7	9	10
21	8	9	11	7	9	10
22	8	10	11	8	9	11
23	9	10	12	8	9	11
24	9	10	12	8	10	11
25	9	10	12	8	10	12
26	9	11	12	9	10	12
27	10	11	13	9	10	12
28	10	11	13	9	11	12
29	10	12	13	9	11	13
30	10	12	14	10	11	13
31	11	12	14	10	11	13
32	11	12	14	10	12	13
33	11	13	14	10	12	14
34	11	13	15	10	12	14
35	11	13	15	11	12	14
36	11	13	15	11	13	15
37	12	14	16	11	13	15
38	12	14	16	11	13	15
39	12	14	16	12	13	15
40	13	14	16	12	14	16
41	13	15	17	12	14	16
42	13	15	17	12	14	16
43	13	15	17	12	14	17
44	13	15	17	13	15	17
45	14	15	18	13	15	17
46	14	16	18	13	15	17
47	14	16	18	13	15	18
48	14	16	18	14	15	18
49	15	16	19	14	16	18
50	15	16	19	14	16	18
60	17	19	21	16	18	21
70	19	21	24	18	21	23
80	21	24	26	20	23	26
90	23	26	29	22	25	28
100	26	28	31	24	27	30

7. *Nat. Bureau of Standards,* Applied Mathematics Series 6, Tables of the binomial probability distribution. U. S. Gov't Printing Office, Washington, D. C., 1950.
8. PERYAM, D. R., AND SWARTZ, V. W. Measurement of sensory differences. *Food Technol.* **4**, 390 (1950).
9. ROESSLER, E. B., WARREN, J., AND GUYMON, J. F. Significance in triangular taste tests. *Food Research,* **13**, 503 (1948).
10. ROMIG, H. G. *50–100 Binomial Tables.* 1952, Wiley, New York.
11. WOOD, E. C. Organoleptic tests in the food industry. III. Some statistical considerations in organoleptic tests. *J. Soc. Chem. Ind.,* **68**, 128 (1949).

STUDIES ON THE CHEMISTRY OF CURED MEAT PIGMENT FADING[a]

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Since the advent of self service merchandising of pre-packaged meats, fading or discoloration of cured products has become a problem to the meat packing industry. This undesirable color change involves the nitric oxide derivative of either myoglobin or denatured myoglobin. In practice, many cured meat products are heated during their manufacture. On heating, the protein undergoes denaturation and becomes water insoluble. This heated pigment has been assumed to be denatured globin nitric oxide myohemochrome. Indirect evidence presented here is in agreement with this idea. The purified heat denatured pigment was used in this work in order to find out what changes light and oxygen may produce in the pigment itself.[c]

Excluding biological factors, such as bacteria, and biochemical ones such as enzymes, the major factors known to cause discoloration are oxygen, light, and dehydration. Two of the important processes involving oxygen either directly or indirectly are the oxidation of nitric oxide and the oxidation of the heme iron of the pigment.

George and Stratmann *(4)* found that a total of 2.5 ± 0.3 moles of oxygen were required for the oxidation of one mole of myoglobin to metmyoglobin. This is much more than would be expected from the single electron transfer required in oxidizing the ferrous iron to the ferric form. These authors proposed a mechanism for oxidation of the iron which involves formation of peroxides and subsequent oxidation of the heme iron. It was thus desirable to find out if oxidative processes occurring during fading could be related to this work. As Watts *(15)* recently noted, the nitric oxide derivative can be regenerated in meat in the presence of nitrite salts and reducing agents such as sulphydryl groups produced on protein denaturation. Thus, it is of interest to know what happens to the nitric oxide during fading. It is also of interest to know whether or not oxygen and light are involved in further oxidations of the hemichrome since a change might affect the ability to regenerate color. The possibility was explored that some particular component of cured meat might be involved in destruction of the hemichrome.

The identity of the pigment. The pigment in question is a solid, and therefore it is possible to prepare it free of soluble reducing agents and nitrite salts; a simpler system can thus be studied.

An attempt was made to obtain evidence on the identity of the cured meat pigment by means of reflectance spectrophotometry. However, samples faded during preparation and this method was abandoned.

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[c] Nomenclature used in this paper is that of Lemberg and Legge *(7)*.

EXPERIMENTAL

To obtain spectral curves, a thin layer of the wet pigment was compressed between two glass plates made from standard microscope slides (1.10 mm. thick) cut into four equal rectangles. This was done in a vise and pressure was maintained for 30 minutes. The resulting samples were nearly transparent. Absorbance was determined, using a Beckmann Model DU spectrophotometer.

Purified metmyoglobin was heated to 85°C. for 15 minutes and was washed twice by centrifugation. The solid had a strong absorption peak at 532 mμ and a very small peak at 558 mμ. This corresponded to that of the hemichromes *(3, 7)*.

A sample of denatured globin nitric oxide myohemochrome completely free of the myohemichrome was not obtained. The observed adsorption spectra could be interpreted as being a mixture of the denatured globin myohemichrome and denatured globin nitric oxide myohemochrome assuming the latter has an adsorption spectrum similar to nitric oxide myoglobin with peaks at 544 and 575 mμ as reported by Ginger and Schweigert *(5)*. After irradiation of the sample for 500 minutes in the Warburg flasks under the lighting conditions described later, the absorption curve was nearly identical to that for the myohemichrome prepared by heating purified metmyoglobin.

Preparation of Warburg samples. Since oxygen is known to be involved in fading as witnessed by the increased stability of vacuum packaged cured meat products, it was desirable to relate oxygen uptake and any other observable change such as production of nitrite. For this purpose manometric methods were used.

The pigment studied, denatured globin nitric oxide myohemochrome, was prepared from purified metmyoglobin isolated by a combination of the methods of Theorell *(13)* and Morgan *(10)*. This procedure was somewhat similar to that described by Ginger and Schweigert *(5)*.

Twenty lbs. of beef round was ground in a meat grinder and held over night with one part water and was then filtered. Part of the unwanted protein was precipitated with basic lead acetate. Hemoglobin was removed by making the solution 3 molar in potassium phosphates as described by Morgan *(10)*. The extract was dialyzed against cold distilled water to remove phosphate and then against saturated ammonium sulphate. The precipitate was centrifuged off and was extracted with 82% saturated ammonium sulphate until a slight change of color of the small amount of remaining protein was noted. This solid was discarded. The ammonium sulphate was removed by dialysis with mechanical agitation. Metmyoglobin in the dialyzed purified material was determined as cyanmetmyoglobin. The millimolar extinction value for cyanmethemoglobin of 11.5 given by Drabkin and Austin *(2)* was used for determination of the myoglobin in this and all other samples.

Samples of denatured globin nitric oxide myochemochrome were prepared for the Warburg experiments as follows:

A quantity of metmyoglobin sufficient to give a final concentration of 0.4 micromoles per ml. was measured into a 100 ml. flask. Sodium nitrite (10 ml. 0.1%) and sodium hydrosulphite (10 ml. 2%) were added and the volume was made up to 100 ml. with water. Low concentrations of reagents were chosen to minimize undesirable protein oxidation. Aliquots of 5 ml. each containing approximately 2 micromoles of myoglobin were heated in 15 ml. centrifuge tubes for 15 minutes in an 85°C. water bath. The samples were centrifuged. The red precipitate was washed 6 times, as quickly as possible, with 4°C. distilled water by centrifugation. This was done in a cold room in dim light.

The precipitate was quickly quantitatively transferred to Warburg flasks by mixing with a portion of 0.5 M. pH 6.20 phosphate buffer sufficient to make 2 ml. of slurry. The slurry was removed to the Warburg flask with a 2 ml. pipette. A second, approximately 2 ml., portion of buffer was added; this was removed with the same pipette. One ml. of 5.0% NaCl was then added to the centrifuge tube and the 2 ml. pipette rinsed before the solution was transferred to the Warburg flasks. In this manner, it was possible to know the volume of material in the flask although the amount of phosphate buffer was not exactly known.

When it was desired to prepare the hemichrome, the same procedure was used except no sodium nitrate or sodium hydrosulphite was added before heating.

A seven-position Warburg apparatus was specially constructed for this work, using a rectangular glass tank. It differed from conventional equipment only in the fact that

shaking was more rapid (180-200 strokes per minute with short strokes). This rapid shaking, aided by a glass bead, prevented the pigment from settling. The bottoms of conventional conical Warburg flasks were illuminated by a system comprising a mirror set at 45° to the horizontal in the bath and a type A-H6 General Electric high pressure water jacketed mercury arc lamp. The light was restricted to the visible range with a Corning glass filter No. 3389 of 3.0 mm. thickness to cut off the ultraviolet end of the spectrum at 430 mμ, and an Ansco heat filter to remove the infrared. The light path from the mercury tube to the flasks was approximately 24 inches. The illumination was held constant by slightly shifting the lamp to sample distance to give the same reading (350) on a Weston photographic light meter held just above the bath over the sample positions. The absolute light intensity was determined by the use of the Warburg, Schocken *(14)* actinometer. Protoporphyrin, prepared from blood hemoglobin *(6)*, was used as the sensitizer. For this determination, each Warburg flask contained 10 mg. protoporphyrin, 200 mg. thiourea, and 5 ml. pyridine. In this case, the thermobarometer contained 5 ml. of pyridine.

When nitrate and nitrite production was being studied, the light intensity was equivalent to 350 on a Weston light meter and corresponded to 1.8 micromoles quanta per minute per flask. When it was desired to carry out the runs in the dark, the flasks were wrapped with aluminum foil.

Color scoring was carried out for some samples by use of the freshly prepared nitric oxide myohemochromogen as a standard arbitrarily given a score of 10. For the 0 score, the myohemichrome was prepared by heating purified metmyoglobin. Color scores less than 0 were used when the sample was visually judged to be below the myohemichrome standard in redness. Both the 0 score and the 10 score standards were placed in Warburg flasks for comparison with the irradiated samples. For this observation, the samples were quickly removed from the bath, compared with the standards, and replaced.

At the end of the Warburg runs, the suspensions of pigment were removed with a pipette. The flasks were rinsed in small portions of water. The washings were combined with the suspension. The suspension was quickly centrifuged and was washed twice with cold water.

The supernatant liquid was made up to a volume of 50 ml. with water. Aliquots of 15 ml. each were taken for nitrite analysis and 30 ml. samples were used for the nitrate analysis.

Nitrite expressed as NO_2^- was determined by the method of Shinn *(11)*. In order to obtain the 2 ml. sample required in the nitrate determination, 0.1 ml. of 0.1% sodium hydroxide solution was added to the 30 ml. aliquots. The aliquots were evaporated just short of dryness on a steam bath. The sides of the test tubes were washed down with a small amount of water. The sample was evaporated just to dryness. Ammonium sulfamate solution (1 ml. of 0.5%) was added to prevent interference by nitrite and 1 ml. of water was added to give the 2 ml. of solution required in the nitrate determination. Nitrate was then determined by the brucine sulfuric acid method of Mellette, Brodsky, and Palmer *(9)*.

In five of the Warburg runs comprising 24 samples, irradiation was carried out for approximately 350 minutes and nitrate and nitrite were determined. All of the Warburg experiments were carried out at 38° ± 0.3°C.

In following the course of nitrite and nitrate production, samples were irradiated with and without KOH in the wells. Individual samples were irradiated from 31 minutes to 434 minutes each, with observation of oxygen uptake.

At the end of the irradiation period, the contents of the flask wells were analyzed for nitrate or nitrite.

Some Warburg runs without the benefit of nitrite and nitrate analysis were carried out under essentially the same conditions as described above except in some cases small amounts of oleic acid were added. Oxygen uptake was observed in the light, in the dark, in air (148 m.m. pO_2) and in some cases in pure oxygen (742 m.m. pO_2). The amount of metmyoglobin used for preparing samples, determined for each experiment as cyanmetmyoglobin, was the same for each of the samples in a Warburg run, but the amount varied between 1.8 to 3.13 for different runs. Light intensities between 200 and 400, as measured by the Weston meter, were used.

An attempt was made to demonstrate the presence or absence of hydrogen peroxide through its reaction with pyruvic acid to form CO_2 *(16)*. Oxygen uptake was observed

for control samples with KOH and without KOH compared to samples containing 1 ml. 3.33% sodium pyruvate without KOH in the wells. All samples were in pH 6.20 phosphate buffer.

In order to observe the effect of extraneous proteins on the fading reaction, several experiments were conducted in which metmyoglobin was prepared simply by heating a water extract of meat to about 68°C. until some of the metmyoglobin began to precipitate. The solution was quickly chilled and the metmyoglobin was determined as cyanmetmyoglobin.

It was desired to find out if any of the nitric oxide is lost as nitrogen during fading, through a possible reaction of the free amino groups of the protein with nitric oxide. An experiment was carried out with samples of the heat denatured nitric oxide myochemochrome prepared from 4.3 micromoles per flask of metmyoglobin prepared by heat fractionation of a water extract of beef. Samples were irradiated 225 minutes in the light and in the dark with nitrogen freed of traces of oxygen by passing it slowly through alkaline pyrogallol. In all cases, KOH was used in the wells. Control samples were irradiated in the light and in the dark with air. Gas changes were observed for 225 minutes.

Oxygen uptake was also observed for several samples of the hemichrome prepared from purified metmyoglobin.

RESULTS

Based on recovered nitrite and nitrate and on the light adsorption curves obtained for the solid pigment all samples used in this work contained some of the myohemichrome at the start of Warburg runs.

Extended oxidation of the purified denatured globin nitric oxide myohemochrome in pure oxygen, using the usual illumination, gave gas uptake curves of Figure 1.

These curves show the peculiar shape in the first 100 to 200 minutes often observed in these experiments.

In the second stage, photochemical and non-photochemical oxygen using processes occurred. A gas, soluble in KOH and assumed to be CO_2, was given off in the light and in the dark. Light increased both the rate of oxygen uptake and CO_2 production.

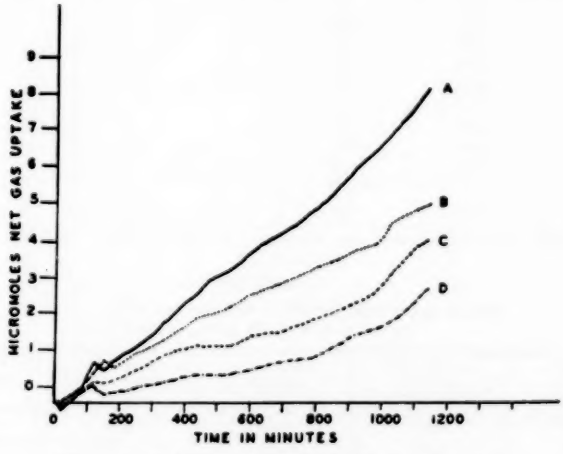

Figure 1. Rate of net gas uptake in pure oxygen.

A. Light KOH
B. Dark KOH
C. Light no KOH
D. Dark no KOH

In the presence of sodium pyruvate additional carbon dioxide production was not observed. This suggests that hydrogen peroxide production may not be involved. It should be noted that at pH 6.2 in approximately 0.04 M. phosphate buffer, quantitative evolution of CO_2 would not be expected.

As Table 1 shows, two major products of the oxidation of denatured globin nitric oxide myohemochrome were the nitrite and nitrate ions. Since the starting amount of pigment was 2.00 micromoles and since the results represented 5 different runs at different times, it appeared possible that nitrite was also lost as some other product. It is evident from these results that nitrate or nitrite found in the KOH represents loss of NO as a gas, either in the form of NO or the form of NO_2.

Under the specific conditions of these experiments, based on the starting amount of metmyoglobin, the average quantum yield for the reaction was estimated to be .0016, or based on the amount of nitrate and nitrite recovered the average quantum yield was estimated to be .0027.

Table 2 shows that nitric oxide was lost with the formation of nitrite and nitrate in the dark as well as in the light.

In Figure 2 the color score and the oxygen uptake curves are given for the same Warburg run as that of Figure 3 showing the rate of nitrite and nitrate production.

Together the curves in these two figures showed that at about 200 minutes under the conditions used, a change occurred in the character of the oxidation in which nitrate production decreased, rate of color loss increased slightly, gas uptake rate decreased, and rate of production of CO_2 increased somewhat. Figure 4 shows the effect of light

TABLE 1

Nitrate and nitrite produced and oxygen taken up in the presence of light with KOH in the wells[1]

Micromoles nitrite in supernatant	Micromoles nitrate in supernatant	Micromoles nitrite wells	Micromoles nitrate wells	Micromoles oxygen uptake at time T	Minutes total irradiation time
.10	0.59		.20	1.00	362
.19	0.70		.21	1.00	362
.19	0.45		.16	1.07	362
0.37	0.56	.15		1.11	332
0.35	0.68	.16		1.11	332
0.33	0.76	.10		1.25	332
0.24	0.55	.02		.76	350
0.22	0.72	.16		.87	350
.16	0.77	.14		1.00	434
0.13	0.60	.11		.63	183
0.12	0.38	.07		.36	110
0.07	0.20	.05		.26	45
Averages					
0.21	0.58	.11	.19	.88	296

[1] The sample was prepared from 2.00 micromoles of metmyoglobin in each case.

TABLE 2

Nitrate and nitrite produced and oxygen taken up in the dark with KOH in the wells[1]

Micromoles nitrite in supernatant	Micromoles nitrate in supernatant	Micromoles nitrite wells	Micromoles oxygen uptake at time T	Minutes total irradiation time
0.080	0.44	.03	0.44	350
0.190	0.40	.09	.67	340
0.074	0.40	.11	.54	508
Averages				
0.11	0.41	.08	0.55	

[1] The sample was prepared from 2.00 micromoles of metmyoglobin in each case.

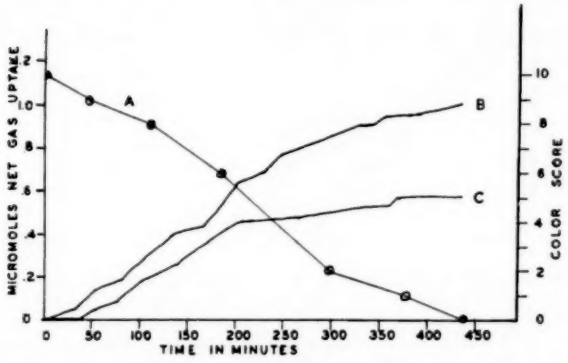

Figure 2. Rate of net gas uptake and rate of color loss in air.
A. Color score
B. KOH in wells
C. No KOH

on the rate of fading as judged by visual scoring. It also shows that fading occurred in the dark.

For samples irradiated 250 minutes or longer, the average amount of nitrate and nitrite recovered was estimated to be 60% based on the metmyoglobin used in preparing the sample with a maximum value of 65%. The ratio of nitrite to nitrate for 12 samples without KOH in the wells was .50, Std. Dev. = ± .15 with a maximum value of .77.

Figure 5 shows the oxygen uptake curve for one of the Warburg experiments carried out without analysis of the flask contents.

The rate of CO_2 production varied somewhat, as compared to the net gas uptake, from one experiment to another and was often less than the run of Figure 5.

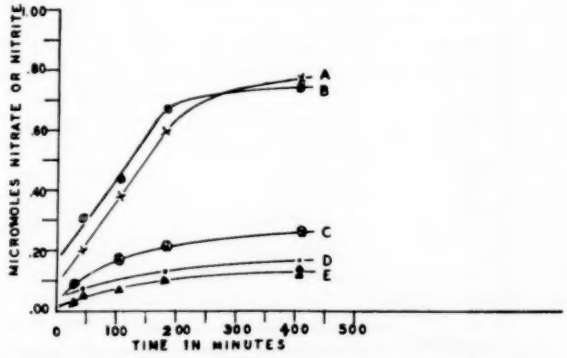

Figure 3. Rate of nitrate and nitrite production during the irradiation of nitric oxide myohemochromogen in strong light.
A. Nitrate KOH in the wells
B. Nitrate no KOH
C. Nitrite no KOH
D. Nitrite KOH in wells
E. Nitrite in the KOH

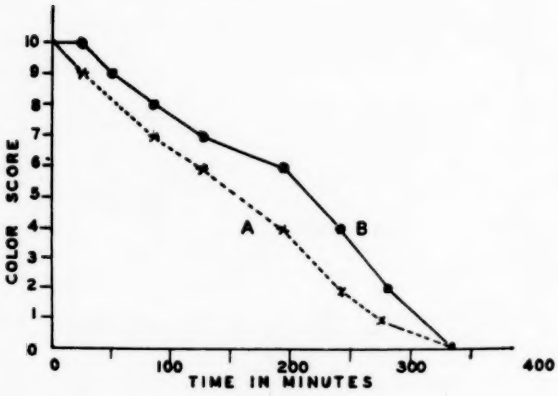

Figure 4. Rate of color loss.
A. In strong light
B. In the dark

The Warburg curves were often characterized by a period of constant or slightly accelerating rate up to about 200 minutes in light and air, then usually for a short time the rate dropped to 0 and after a few minutes increased. This effect was noted in somewhat different degrees for many of the samples studied.

It is not seen here, but there was often a small amount of gas corresponding to 0.1-0.2 micromoles given off in the first few minutes. This effect could be due to adsorbed gas.

In the experiment with a nitrogen atmosphere, in 225 minutes at 26.5°C., 20.7 microliters of gas were given off in the light. In the dark, 24.0 microliters of gas production was observed. In air and light, the gas (oxygen) uptake was 58.0 and 48.0 microliters. In air and dark, gas uptake amounted to 6.2 microliters.

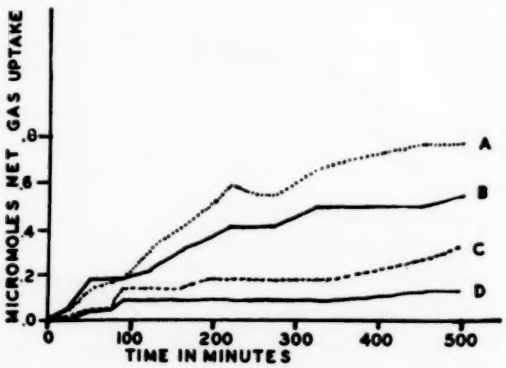

Figure 5. Rate of net gas uptake in air.
A. Light KOH
B. Dark KOH
C. Light no KOH
D. Dark no KOH

TABLE 3

Average nitrate and nitrite production and oxygen consumption in manometric experiments

Treatment	No.	Micromoles in supernatant		Micromoles in KOH		Micromoles net gas uptake	Minutes total irradiation time T
		Nitrite	Nitrate	Nitrite	Nitrate		
Light, KOH	12	0.21	0.58	0.11	0.19	0.88	296
Dark, KOH	3	0.11	0.41	0.08	.....	0.55	483
Light, no KOH	12	0.27	0.54	.....	.....	0.50	298
Dark, no KOH	2	0.19	0.29	.....	.....	0.24	430

Samples prepared by heat fractionation of water extracts of beef showed a depressed oxygen uptake rate in the first part of the run. The curves were similar in shape to those obtained with the purified pigment. A depressed rate in the first part of the runs suggests the possibility that nitrogen might be given off.

The net gas uptake value for the heat denatured globin myochemichrome with KOH in the wells in 296 minutes, was 0.40, 0.62, 0.50, 0.0, and 0.13 microliters, respectively (ave. = .33 microliters). With no KOH in the wells the average net gas uptake was 0.06 microliters in 296 minutes. This compares with an average net gas uptake of .88 microliters for the nitric oxide derivative in the same time with KOH in the wells.

DISCUSSION

There are several possible mechanisms that can be proposed to account for the results found in these experiments, any one of which could be related to practical questions on meat discoloration.

Color stability may be related to reformation of the nitric oxide derivative by reducing materials. The loss of nitrite could become a limiting factor in color protection. Thus the question, what happens to the nitric oxide, becomes of practical importance.

Possible explanations include, first, possible participation of nitrite in the oxidation of the iron with subsequent formation of nitrate. The second possibility is that a cyclic reformation of the nitric oxide myohemochrome takes place in the presence of reducing activity associated with protein. Thirdly, a Van Slyke reaction between protein free amino groups and nitric oxide may be involved.

The nitrate and nitrite recovered in the supernatant liquid was never over 65% of the molar amount of metmyoglobin used in preparing the original sample, thus some loss of nitric oxide may have occurred during the preparation of the sample.

Part of the apparent nitric oxide loss may have been due to a lower affinity of the denatured globin myohemochrome for nitric oxide than is the case for myoglobin. Lemberg and Legge *(8)* mention that the affinity of denatured globin hemochrome for carbon monoxide is about one-half that of hemoglobin for carbon monoxide.

Several suggestions can be made in line with the observation that there is a consistent break in the oxygen uptake curves, that nitrate production breaks sharply and that more nitrate than nitrite is produced. One possibility is that nitrite itself may be directly concerned in the oxidation of the heme iron. This is in line with the rather low oxygen uptake values observed compared to the theoretical oxygen uptake required to produce products. Oxidation of the hemochrome by nitrite however might be ex-

pected to persist until all of the nitric oxide derivative is oxidized. A somewhat more likely possibility is that even with the thoroughly washed solid pigment some reducing activity remains associated with the protein which can result in regeneration of the nitric oxide derivative. In this case, oxygen uptake might be expected to continue at a relatively high rate until all of the reducing activity is used up; then the oxygen uptake would be expected to drop. This scheme could well account for the fact that the ratio of nitrite to nitrate is less than one since production of excess nitrate over nitrite would be expected until all of the reducing activity is used up.

The sharp break in the curve for production of nitrate before all of the color is lost indicates that some active process, occurring only during the early part of the oxidation, may be involved. This behavior is also in line with the possibility that a cyclic regeneration of the nitric oxide myochemochrome takes place in the presence of reducing activity.

This idea is in line with the possibility that preservation of color may be an active process depending on regeneration of color. In this case, the cured color would be expected to persist until one or more of the following conditions occurs. 1. The reducing activity is used up. 2. The nitrite is gone. 3. In the absence of remaining reducing agent all of the pigment is oxidized to the hemichrome. 4. Oxidation of the hemichrome beyond the stage of its ability to reform color takes place.

A reaction between nitric oxide and free amino groups could give the low observed oxygen uptake values. The possibility of such a reaction leading to loss of nitrite cannot be ignored since this could account for many of the observations. At a pH value of 6.2 and with a nitrite concentration of the order of 4 p.p.m. a Van Slyke reaction would probably not occur to a measurable extent. It has been shown that the rate of this reaction depends on the concentration of the free acid *(12)*. Such a reaction is suggested by the observation that a gas was given off when samples were shaken in a nitrogen atmosphere.

The rate curves can possibly be interpreted to be a summation of a normal decreasing rate curve for oxygen uptake and a decreasing rate curve for nitrogen production. If this is so then the break in nitrate production might be explained by the consideration that as the reactant disappears the oxygen uptake decreases but nitric oxide temporarily persists in the solution.

Destruction of the hemichrome. It was considered possible that some component of cured meat might be involved in oxidative processes that could cause destruction of the hemichrome and that discoloration of cured meat might involve oxidative processes beyond the changes associated with the loss of nitric oxide from the myohemochrome.

Many components that might conceivably be involved in such an oxidation were shaken with 0.2-0.3 micromole samples of denatured globin myochemichrome in 5 ml. of water and the rate of color loss was observed in the light and in the dark. The samples were shaken rapidly at about 200 strokes per minute under tungsten (400 Weston) illumination using a heat filter.

Since the pigment is a red brown color, for exploratory experiments, visual observation of the color was used as an indication of undesirable changes.

The amino acids, cysteine, tryptophan, methionine, tyrosine, and glycine (1 ml. of a 1% solution of each), iron 100 p.p.m., methyl oleate 30 mg., NaCl 5%, and phosphate buffer 0.05 M., pH 5.70, 6.04, and 7.20 did not increase the rate of discoloration in the light. Oleic acid and rancid fat both caused color loss, oleic acid freed of linoleic acid by low temperature crystallization *(1)* also caused an increased rate of color loss. Methyl laurate (0 iodine number) and methyoleate did not. Lauric acid (0 iodine number) dissolved in 3 parts methyl laurate was as effective in destroying color as was purified oleic acid in 3 parts methyl laurate. With fatty acids (lauric and purified oleic acids), color was rapidly destroyed when shaken in the light, but not when shaken in the dark.

It was noted that after shaking with fatty acids the colored part had to a large extent become separated from the protein. These experiments indicate that the heme group (possibly hematin) may have been split from the hemichrome by free fatty acid and that a light induced reaction occurs resulting in the production of a dark brown unidentified material. The light induced change in the presence of free fatty acids may possibly be related to the darkening often associated with fading in cured meat products.

CONCLUSIONS

In the presence of visible light and air or air alone, at least part of the nitric oxide of purified heat denatured globin nitric oxide myohemochrome is further oxidized to give the nitrite and nitrate ions.

Nitric oxide from the pigment of cured meat can be lost as a gas, possibly in the form of nitric oxide or possibly in the form of nitrogen dioxide.

A gas which is adsorbable by potassium hydroxide solutions and which is probably CO_2, is produced in the oxidation of the hemichrome in the air and in the presence of high intensity visible light.

Oxygen adsorption is associated both with processes involving loss of nitric oxide from the pigment and the oxidation of the resulting hemichrome.

Several possible pathways have been suggested for the nitric oxide lost from denatured globin myohemochrome which could be of practical significance to the problem of cured meat discoloration.

Denatured globin hemichrome shaken in the presence of free fatty acids undergoes a darkening not observed in the light. Oxidation of the hemichrome may possibly be of significance in cured meat color deterioration.

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LITERATURE CITED

1. Brown, J. B., and Shinowara, G. Y. Studies on the chemistry of fatty acids VI. The preparation of pure oleic acid. *J. Amer. Chem. Soc.*, **59**, 6-8 (1937).

2. DRABKIN, D. L., AND AUSTIN, J. H. Spectrophotometric studies II. Preparation from washed blood cells; Nitric oxide hemoglobin and sulfhemoglobin. *J. Biol. Chem.*, **112**, 51-65 (1935-36).
3. DRABKIN, D. L., AND AUSTIN, J. H. Spectrophotometric studies IV. Hemochromogens. *J. Biol. Chem.*, **112**, 89-104 (1935-36).
4. GEORGE, P., AND STRATMANN, C. J. The oxidation of myoglobin to metmyoglobin by oxygen II. The relation between the first order rate constant and the partial pressure of oxygen. *Biochem. J.*, **51**, 418-425 (1952).
5. GINGER, I. D., AND SCHWEIGERT, B. S. Biochemistry of myoglobin. Chemical studies with purified metmyoglobin. *Agr. and Food Chem.*, **2**, 1039-1040 (1954).
6. GRINSTEIN, M. Studies of protoporphyrin VII. A simple and improved method for preparation of pure protoporphyrin from hemoglobin. *J. Biol. Chem.*, **167**, 515-519 (1947).
7. LEMBERG, R., AND LEGGE, J. W. *Hematin Compounds and Bile Pigments.* 1949. Interscience Publishers, page 223.
8. LEMBERG, R., AND LEGGE, J. W., *ibid.*, page 196.
9. MELLETTE, S. J., BRODSKY, W. A., AND PALMER, L., JR. A rapid modification of the brucine method for determination of nitrates in biological fluids. *J. Lab. Clin. Med.*, **41**, 963-967 (1953).
10. MORGAN, V. R. Studies on myoglobin I. The solubility of myoglobin in concentrated ammonium sulphate solution. *J. Biol. Chem.*, **112**, 557-563 (1936).
11. SHINN, M. Colorimetric method for determination of nitrite. *Ind. Eng. Chem., Anal. Ed.*, **13**, 33-35 (1941).
12. TAYLOR, T. W. F. The action of nitrous acid on amino compounds II. Aliphatic amino acids. *J. of Chem. Soc. of London*, 1897-1906 (1928).
13. THEORELL, H. Kristallinisches myoglobin I. Kristallisieren reinigung des myoglobin sowie vor laufige mitteilung uben sein molekulargewicht. *Biochem. Ztschr.*, **252**, 1-7 (1923).
14. WARBURG, O., AND SCHOCKEN, V. A manometric actinometer for the visible spectrum. *Archives of Biochem.*, **21**, 363-369 (1949).
15. WATTS, B. M., ERDMAN, A. M., AND WENTWORTH, J. Relation of free sulphydryl groups to cured meat color. *Agr. and Food Chem.*, **3**, 147-151 (1955).
16. WEIL, L., GORDON, W. G., AND BUCHERT, A. R. Photooxidation of amino acids in the presence of methylene blue. *Arch. Biochem. Biophys.*, **33**, 90-109 (1951).

SORBIC ACID AS A FUNGISTATIC AGENT FOR FOODS XI. EFFECTIVENESS OF SORBIC ACID IN PROTECTING CAKES[a]

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Reports have appeared on the harmlessness *(6, 7, 23, 24)*, purity *(15, 22, 9)*, determination *(12, 13)*, and fate *(14, 15, 16, 9)* of sorbic acid in foods. The mechanisms by which sorbic acid inhibits mold growth have also been intensively explored *(16, 8, 9)*. In addition to the demonstrations that sorbic acid is of value in protecting cheese *(22, 23)*, margarine *(20)*, fish *(3)*, pickles *(18, 10, 5, 2, 21)*, gum and sugar solutions *(19)*, citrus products *(17)*, and fruit purees *(1)* against yeast and mold spoilage, the use of sorbic acid on an expanding commercial scale for the protection of other food products *(4)* including chocolate syrups, refrigerated fresh fruit salads, potato salads, and cold slaw attests to the effectiveness of sorbic acid in protecting foods in general.

Two factors have been largely responsible for the growing preferential use of sorbic acid over the other fungistatic agents currently allowed by the U. S. Food and Drug Administration for use as food supplements; these are effectiveness and flavor. Not only has sorbic acid proved to be more effective than benzoic or propionic acid in these applications *(3, 19, 20, 21, 22)* but the flavor of the resulting foods is not impaired. This difference in flavor quality is due to the need to use on a weight for weight basis less sorbic acid and to the characteristic flavors of benzoic and propionic acid particularly in acid foods. Sorbic acid cannot be used to conceal damage or inferiority in foods; use of this fungistatic agent in plant operations will not permit relaxation in good sanitary practices *(16, 23)*. Sorbic acid can be metabolized by molds and quantity thereby reduced, if the ratio of molds to initial sorbic acid concentration is high *(16)*.

The present report summarizes studies on the value of sorbic acid relative to that of propionate in protecting cakes against mold spoilage. Mold spoilage is not as serious a problem in cake merchandizing as it is in the case of bread and rolls although the pH of cakes make them more conducive to support mold growth. The higher sugar content, lower moisture, and the use of icings minimize the mold problem in cakes. Mold spoilage becomes serious, however, when cakes are on sale for extended periods, particularly during the summer months, and when the products are of a relatively higher moisture content. Fruit cakes which have long merchandising periods must also be protected. It has become common practice for the commercial baker to add sodium or calcium propionate in amounts varying from 3 to 5 ounces per 100 pounds of batter (0.1875 to 0.3125%) and at times as much as 7 ounces (0.44%) depending upon the type of cake. In a few bakeries, notably in Canada, sodium benzoate is used.

[a] Most of the laboratory work reported in this paper was conducted in the laboratory of Bohn Food Research, Inc., New York, and was supported by a grant from The Best Foods, Inc., New York.

There has been a need for a more effective, non-toxic, fungistatic agent for cakes—one that would not affect the delicate flavor of cakes. The results of studies summarized in this report point to the value of sorbic acid in fulfilling this need. Following the completion of the present studies, the U. S. Food and Drug Administration advised *(11)* that it had no objections to the use of sorbic acid as a mold inhibitor in cakes.

EXPERIMENTAL

Cake making methods. Cake mixes were prepared containing all the ingredients required to make several types of cakes. To each was added water to form the batter. The mold inhibitors, when added, were either suspended or dissolved in the water or suspended in the shortening component; in every case concentrations have been expressed on a batter weight basis. In Table 1 are listed the cake batter formulas employed in the present laboratory studies.

TABLE 1

Batter formulas employed in the current laboratory studies

Ingredient	White cake	Yellow cake	Devil's Food cake
	Parts		
Flour	38.0	35.0	32.5
Sugar	42.9	43.0	41.5
Shortening	12.0	13.0	12.0
Milk solids, non-fat	3.0	3.0	3.0
Egg white (dry)	1.15	....	....
Egg, whole (dry)	.....	3.0	3.5
Cocoa	.....	....	5.0
Salt	1.20	1.0	1.0
Baking powder	1.60	2.0	0.5
Baking soda	.....	....	1.0
Sodium acid pyrophosphate	0.15	....	....
Dry mix	100.00	100.00	100.00
Water	50.0	50.0	50.0

The cake batters were scaled at 12 ounces in round layer cake pans, measuring 8 inches in diameter. The layers were baked at 375-400°F. for about 20 minutes.

Mold inoculation and incubation methods. Most tests involved deliberate inoculation of the cakes with mixed mold cultures. Slices of moldy yellow cake were first air-dried and then pulverized. The mixed mold cultures thus produced consisted of the common air-borne molds associated with bread and cake spoilage and included species of Aspergillus, Penicillium, and Rhizopus. The use of the former two species assures the presence in the inoculum of those molds shown to exhibit the greatest tolerance toward sorbic acid *(1)*. A 0.20 gram portion of the moldy cake powder was thoroughly dispersed in 500 ml. of water, the dispersion filtered through cotton into a sprayer equipped with a rubber bulb. Three squirts from a distance of two feet were then used to inoculate the surface of each cake with a standard spray.

Two methods of incubation of the inoculated cakes were employed for the most part. In one method, circles were cut from the cakes and these were incubated with inoculated crumb surface uppermost in closed Petri dishes; the dishes were stored under air and over water at 85°F. In the other method, squares of the cakes after mold inoculation were placed with crust surface uppermost in closed canisters. The latter contained a beaker of water to provide an atmosphere of air of 100% relative humidity in direct contact with the cakes. The sealed assemblies were incubated at 85°F. Extent of mold growth has been indicated by "—" for no growth and up to "++++" for heavy growth.

Physico-chemical tests. The cakes were analyzed gravimetrically for moisture content, involving first air drying and then dehydration of a representative aliquot at 130°C. for one hour, for pH value using the Beckman pH meter in testing a 10% dispersion of the cake in distilled water, and spectrophotometrically for sorbic acid content *(12, 13)*.

RESULTS

Retention of sorbic acid during cake baking. The spectrophotometric method for the determination of sorbic acid *(13)* has been shown to be applicable to the determination of this fungistatic agent in cakes *(12)*. Indeed, the cakes employed in the study devoted to sorbic acid methodology *(12)* were the same products obtained in the course of the present investigation. The results of tests conducted on cakes made from batters with and without added sorbic acid are summarized in Table 2; these show that there is no

TABLE 2

Retention of sorbic acid during cake baking

Cake	Weight of layer			Sorbic acid		
	Batter	Cake	Loss	Added to batter	Found in cake	Recovery of that added[1]
	gm.	*gm.*	%	%	%	%
White	340	320	5.9	0.200	0.217	102.1
Yellow	340	322	5.3	0.200	0.206	97.5
Devil's Food	340	325	4.4	0.200	0.192	91.8

[1] $\frac{\%\text{ sorbic acid found in cake}}{\%\text{ sorbic acid added to batter}} \times (100 - \%\text{ loss in weight})$

measurable loss of this fungistatic agent during baking. This is surprising since sorbic acid may be readily volatilized by either heat sublimation *(9)* or steam distillation *(13)*. Sorbic acid recoveries in analyzing cakes, following the addition of the fungistatic agent to the batters, are of the same order of magnitude as those noted following the addition of the sorbic acid to the blank control cakes themselves just prior to analyses *(12)*; in both cases recoveries approach theoretical values.

Preliminary studies of fungistatic activity. From the results of these early studies, which are summarized in Table 3, it was immediately apparent that sorbic acid is more effective in protecting white cakes against mold spoilage than either sodium propionate or benzoate. In these preliminary tests, the cake slices were incubated in closed Petri dishes, containing within the top cover a sheet of filter paper, moistened at the time of each examination. The objection to this test was the tendency for the cake to dry unevenly during the incubation period. For this reason, in all subsequent evaluations, the cake slices in Petri dishes, were stored according to the preferred method described earlier in this report. Because of the similarity of results on relative effectiveness of the different fungistatic agents noted in these preliminary experiments involving incubation of the inoculated cakes at 80°F. and at 90°F., it was decided to standardize the holding temperature in all subsequent tests at 85°F. The pH of the control cakes varied from 7.0 to 7.2 and moisture content from 27.7 to 28.2%.

It appeared from the results shown in Table 3 that the same degree of fungistatic protection could be imparted to white cakes by using one-sixth to one-third as much sorbic acid as sodium propionate. Sorbic acid was also about three times as effective as the benzoate in protecting the cakes. The same results were obtained when the studies were repeated on a third series of cakes but with a further dilution (1-5) of the mold suspension used for inoculation purposes.

In the fourth series of preliminary tests the same ratings of relative effectiveness were again obtained, the propionate and benzoate being again dissolved in the aqueous phase but the sorbic acid now supplied as a dispersion in the shortening component[b]

[b] An all-purpose vegetable oil shortening (Veo) was plasticized with 5% of sorbic acid in solution and suspension. Sorbic acid was found to be stable in this shortening even after one year of storage at prevailing room temperatures *(20)*. In using this product to supply gradients of sorbic acid, adjustments were made in ratio of regular to fungistatic shortening, keeping the total quantity of shortening constant.

TABLE 3

Relative value of sorbic acid in protecting white cakes against mold spoilage following inoculation with a mixed mold culture

Supplement[1] to batter; first series		Mold growth after incubation of cakes[2] at 90°F. for number of days indicated				
		2	4	5	7	8
None—	Control	−	++++	++++	++++	++++
Sorbic acid,	0.05%	−	++	+++	++++	++++
	0.10%	−	−	−	+	+++
	0.20%	−	−	−	−	−
Sodium propionate,	0.30%	−	+	++	+++	++++
	0.60%	−	−	+	++	++++
Sodium benzoate,	0.10%	−	++	++++	++++	++++
Second series		**After incubation at 80°F.**				
None—	Control	−	+	++	+++	++++
Sorbic acid,	0.05%	−	+	+	++	+++
	0.10%	−	−	+	+	++
	0.20%	−	−	−	−	+
Sodium propionate,	0.30%	−	−	+	++	++
Sodium benzoate,	0.10%	−	+	++	+++	++++

[1] Supplements added as a dispersion or solution in water during batter preparation.
[2] Cake slices incubated in closed Petri dishes containing within the top cover a sheet of filter paper that was moistened at the time of each examination.

of the mix. The results in this series of tests were not only of confirmatory value, but also indicated that sorbic acid in the shortening employed in batter preparations is completely available for fungistatic protection of the cakes.

The cakes made with the benzoate (0.1%) were similar to the control in odor and taste, while those made with the propionate (0.3 and 0.6%) had a sour flavor. Sorbic acid at the 0.2% concentration could be detected by odor during the baking of the cake but not in flavoring the baked cake itself. At the 0.1% concentration, sorbic acid escaped organoleptic detection. All cakes were similar in appearance, being of standard volume, grain, and texture.

Sorbic acid as a fungistatic agent for yellow cakes. In Table 4 are summarized results obtained in evaluating the effectiveness of sorbic acid and sodium propionate in protecting yellow cakes against mold spoilage. Sorbic acid was found to be about four times as effective as the propionate. As little as 0.05% sorbic acid exhibited measurable fungistasis while cakes with 0.10% were much superior to the blank control cakes in resistance to mold spoilage. The propionate failed at most levels of test, the results with 0.30% being hardly better than those obtained with the controls.

Sorbic acid at the higher concentrations increased baking time slightly and decreased crust color due undoubtedly to a lowering of the pH of the batter. However, the interference with crust color development was not evident at the practical use level of 0.10%. Sorbic acid imparted no odor or flavor to the cakes while the propionate at the higher concentrations imparted its characteristic aroma to the cakes.

Sorbic acid as a fungistatic agent for white cakes. In Table 5 are presented results obtained in evaluating the effectiveness of sorbic acid and calcium propionate in protecting white cakes against mold spoilage. Because of the results obtained in studies with the yellow cakes, it was decided to evaluate the propionate at higher concentrations. The inoculated cakes were subjected to only one type of holding test, direct exposure to 100% relative humidity in sealed cans stored at 85°F.

From the results obtained, it was concluded that sorbic acid is about four times as effective as calcium propionate in fungistatic protection of the white cakes. The

TABLE 4

Effectiveness of sorbic acid in protecting yellow cakes against mold spoilage following inoculation with a mixed mold culture and stored at 85° F.

Supplement[1] to batter		pH of cake	Mold growth on slice in Petri dish after days indicated					Mold growth on cake in humidity can after days indicated				
			3	5	6	8	12	3	5	6	8	12
None—	Control	7.58	−	+	++	+++	++++	+	++++	++++	++++	++++
Sorbic acid,	0.025%	7.55	−	+	++	+++	+++	+	+++	++++	++++	++++
	0.05%	7.40	−	−	−	++	++	−	++	++++	++++	++++
	0.10%	7.13	−	−	+	+	++	−	−	+	++	++++
	0.20%	6.80	−	−	−	−	−	−	−	−	−	+++
	0.30%	6.49	−	−	−	−	−	−	−	−	−	−
Sodium propionate,	0.025%	7.60	−	+	++	+++	++++	+	++++	++++	++++	++++
	0.05%	7.61	−	+	++	+++	++++	+	++++	++++	++++	++++
	0.10%	7.61	−	+	++	+++	++++	+	+++	++++	++++	++++
	0.20%	7.55	−	+	++	++	+++	−	++	++++	++++	++++
	0.30%	7.51	−	+	++	++	++	−	++	++++	++++	++++

[1] Supplements added as a dispersion or solution in water during batter preparations. The moisture content of the cakes after baking averaged 28.5%.

TABLE 5

Effectiveness of sorbic acid in protecting white cakes against mold spoilage following inoculation with a mixed mold culture and storage at 85° F.

Supplement[1] to batter		pH of cake	Mold growth in humidity can after days indicated					
			3	4	5	6	8	12
None—	Control	7.00	−	+	++	+++	++++	++++
Sorbic acid,	0.05%	6.90	−	+	+	++	+++	++++
	0.10%	6.75	−	−	−	+	++	++++
	0.20%	6.40	−	−	−	−	−	++
	0.30%	6.05	−	−	−	−	−	−
Calcium propionate,	0.05%	7.00	−	+	++	+++	++++	+++
	0.10%	6.90	−	+	++	+++	++++	+++
	0.20%	6.75	−	+	+	++	++++	+++
	0.30%	6.55	−	+	+	++	+++	+++
	0.40%	6.30	−	−	−	−	+	+++
	0.50%	6.15	−	−	−	−	−	++

[1] Supplements added as a dispersion or solution in water during batter preparations. The moisture content of the cakes after baking averaged 28.1%.

propionate in these tests on white cakes appeared to be more effective than in the comparable study conducted on the series of yellow cakes (compare results in Table 4 with those in Table 5). This is attributed to the more favorable (lower) pH values of the white cakes.

All white cakes were of standard volume, grain and texture. The flavor of the cakes was not altered by the addition of sorbic acid in concentrations of 0.20% or less. Those containing propionate in 0.20% concentration and more had a characteristic aroma.

Sorbic acid as a fungistatic agent for Devil's Food cakes. This type of cake has a high pH (8.5 to 9.5) in order to develop a characteristic reddish brown crumb color and flavor. It is common practice to use 0.44% propionate (7 oz. per 100 lbs. of batter) for fungistatic protection of such cakes. Tests similar to those used in the previous studies on yellow and white cakes were conducted on the chocolate-flavored cakes. These averaged 28.3% in moisture content after baking and had a pH value of 9.55. The addition of sorbic acid (0.30%) depressed the pH to 9.15, while the added calcium propionate was without effect on the pH value.

The blank control cakes, following inoculation, exhibited mold spoilage of the same degree and at approximately the same rate as the control inoculated cakes employed in the preceding white and yellow cakes studies. However, neither the sorbic acid nor the propionate in the maximal concentrations evaluated (0.30 and 0.50%, respectively) provided adequate mold resistance to the Devil's Food cakes; the test cakes simulated the unsupplemented controls in rapidity and degree of mold propagation.

Effect of pH of cake on relative fungistatic activity. It is well known that both sorbic and propionic acids are more effective fungistatic agents in an acid environment. The results of our studies on the three types of cakes would seem to indicate that this is true in the case of cakes also. In our previous studies pH values were measured but allowed to vary as an uncontrolled variable following supplementation of the batters with sorbic acid or with propionate. Experiments were now carried out to evaluate precisely the influence of pH of cake upon the relative fungistatic activity of the two mold inhibitors.

A series of cakes was prepared using the standard yellow cake mix formula (see Table 1). The fungistatic agents were added with the water used in preparing the batter. Acid or alkaline salts were added to the batters to adjust pH of the cakes to the values desired. In Table 6 are shown the quantities required for the pH adjustments in the absence and in the presence of the fungistatic agents.

In Table 7 are summarized the results obtained in determining the efficacy of sorbic acid, relative to that of sodium propionate, in protecting cakes against mold spoilage with the pH variable now under control.

TABLE 6

Adjustment of pH of yellow cakes containing no added fungistatic agent or added sorbic acid or sodium propionate

pH of cake	Fungistatic agent added		pH adjusting agent	
	Identity	Amount[1]	Identity	Amount[1]
		%		%
5.25—5.35	Control	0.00	Calcium acid phosphate	1.35
	Sorbic acid	0.20	Calcium acid phosphate	1.25
	Sodium propionate	0.20	Calcium acid phosphate	1.35
6.80—6.90	Control	0.00	Calcium acid phosphate	0.25
	Sorbic acid	0.20	None	0.00
	Sodium propionate	0.20	Calcium acid phosphate	0.25
8.10—8.20	Control	0.00	Sodium bicarbonate	0.05
	Sorbic acid	0.20	Sodium bicarbonate	0.20
	Sodium propionate	0.20	Sodium bicarbonate	0.05

[1] Expressed on a batter weight basis.

All cakes containing no added fungistatic agent, became moldy in 3 days regardless of difference in pH. Both sorbic acid and propionate in 0.2% concentration in the acid environment of pH 5.25-5.35 protected the cakes for extended periods. Thus, the added propionate prevented mold growth for a period of 10 days, while the sorbic acid protected the product in excess of the maximal holding period of 14 days.

At the more normal reaction of cakes, pH 6.80-6.90, the added sorbic acid protected the cakes for a period of 11 to 13 days while the propionate prevented mold growth for a period of 4 days. Considering that mold growth did not develop in the control (no fungistatic agent added) cake until after 2 days, fungistatic protection amounted to an additional 2 days with the added propionate and an additional 9 to 11 days with the added sorbic acid, about a 5 to 1 ratio in favor of the sorbic acid.

At the alkaline reaction, pH 8.10-8.20, the greater effectiveness of the added sorbic acid over the propionate was again noted. The relative activity ratio was about 4 to 1 in favor of the sorbic acid based upon period of *extended* protection against mold propagation. Both fungistatic agents were far less effective in cakes of the more alkaline reaction. At the pH values of some Devil's Food cakes, *viz.*, pH 9.0-9.5, neither the sorbic acid nor propionates have proven to be of much value in preventing mold growth.

Use of sorbic acid on a commercial scale to protect cakes against mold spoilage. Marble pound cakes, made from combinations of white and chocolate cake batter containing sorbic acid or sodium propionate, were baked on a commercial scale under the supervision of the authors. Control cakes without added fungistatic agents were also included in the series.

The white cake batters contained 11 oz. of sodium propionate or 2¾ oz. of sorbic acid per 460 lb. of batter. The chocolate batters contained 18 oz. of the propionate or 4 oz. of sorbic acid per 460 lb. of batter. Thus the ratio of sorbic acid to propionate was 1:4 or somewhat less, in accordance with recommendations based upon findings in the preceding laboratory studies. The data in Table 8 summarize the results obtained in this study.

It would have been desirable to have used sorbic acid at the preferred 0.1% level. However, the level of propionate could not be increased without risking organoleptic detection by the consumer, and it was desirable to evaluate sorbic acid versus propionate at levels of equivalence established in the laboratory studies. From the results obtained it was apparent that sorbic acid used at a level of one-fourth of that of propionate was at least as effective as the propionate in protecting the cake against mold spoilage. Both fungistatic agents were far more effective, despite use at the lower concentrations, in protecting the white portion of the cakes. Indeed, both agents at the concentrations employed were of very little value in protecting the chocolate portion of the cake in the laboratory tests. In the case of the whole cakes stored in the humidity cans the

TABLE 7

Effect of pH on the relative fungistatic activity of 0.2% sorbic acid or sodium propionate in protecting cakes against mold spoilage following inoculation with a mixed mold culture and storage at 85°F.

Fungistatic agent added	pH of cake	Mold growth on cake in humidity can after days indicated									
		3	4	5	6	7	8	9	10	11	14
None		+	+++	++++	++++	++++	++++	++++	++++	++++	++++
Sorbic acid	5.25—	−	−	−	−	−	−	−	−	−	−
Sodium propionate	5.35	−	−	−	−	−	−	−	−	++	+++
None		+	+++	+++	++++	++++	++++	++++	++++	++++	++++
Sorbic acid	6.80—	−	−	−	−	−	−	−	−	−	++
Sodium propionate	6.90	−	−	++	++	+++	+++	++++	++++	++++	++++
None		+	+++	+++	++++	++++	++++	++++	++++	++++	++++
Sorbic acid	8.10—	−	−	−	−	+	++	++	+++	++++	++++
Sodium propionate	8.20	−	+	++	+++	+++	++++	++++	++++	++++	++++

TABLE 8

Relative value of sorbic acid in very low concentrations in protecting marble pound cakes[1] produced on a commercial scale and inoculated with a mixed mold culture prior to storage at 85°F.

Fungistatic agent added	Added to batter		pH of cakes	
	White	Chocolate	White	Chocolate
	per cent			
Control	0.000	0.000	7.15	6.93
Sorbic acid	0.038	0.054	6.95	6.93
Sodium propionate	0.150	0.244	7.05	7.00

Fungistatic test	Mold growth on slice in Petri dish after days indicated					
	3	4	5	6	7	10
On white portion						
Control	−	+	++	++	+++	++++
Sorbic acid	−	−	−	−	−	++
Sodium propionate	−	−	++	++	+++	++++
On chocolate portion						
Control	−	+	++	++	+++	++++
Sorbic acid	−	+	+	++	++	+++
Sodium propionate	−	+	++	++	+++	+++

Fungistatic test (whole cake)	Mold growth on cake in humidity can after days indicated					
	3	4	5	6	7	10
Control	+	++	+++	++++	++++	++++
Sorbic acid	−	+	+	++	+++	++++
Sodium propionate	−	++	++	+++	+++	++++

[1] The moisture contents of the cakes after baking averaged 26.1%.

two fungistatic agents extended the mold-free life of the cakes by 33%, mold growth starting first on the chocolate areas.[c]

The slabs of marble cakes were also cut into one-pound segments at the bakery and wrapped in moisture-proof cellophane according to commercial practice. The surfaces of the cakes were exposed during this operation to adventitious inoculation with mold spores that may have been present in the air of the bakery. Six cakes from each bake were held for one month at room temperature and examined periodically. The control cakes and those containing the propionate showed several mold spots, those on the latter cakes being in less advanced stages of growth. The cakes with the sorbic acid were less moldy than those with the propionate. It was concluded by the practical baker in charge of the examination of the cakes that one part of sorbic acid was about as effective as four parts of sodium propionate but that both fungistatic agents should be used at higher levels for a desirably greater degree of fungistasis.

The study of commercial cakes was repeated but this time the sorbic acid concentration in the batter was increased to 4 oz. per 460 lb. of white batter and to 6 oz. per 460 lb. of chocolate batter. Sodium propionate concentrations were not increased since the levels employed were those in common usage in this bakery; management objected to increasing these levels because of fear of jeopardizing consumer acceptance of the cake. This increased the ratio of sorbic acid to propionate to 1:3. The results of the laboratory evaluation of the commercial cakes were confirmatory of those obtained earlier when the cakes were made with a ratio of sorbic acid to propionate of 1:4. In

[c] It has been shown in the preceding laboratory tests (see Table 7) that both sodium propionate and sorbic acid are more effective fungistatic agents as the pH of the cake is reduced. In the marble cake the pH of the white and chocolate portions were the same. Thus, the more rapid growth of molds on the chocolate portion, despite higher concentrations of the fungistatic agents, must be attributed to the chocolate cake providing a richer medium for mold growth following the heavy inoculation.

TABLE 9

Relative value of sorbic acid in moderate concentration in protecting yellow pound cakes[1] produced on a commercial scale and inoculated with a mixed mold culture prior to storage at 86°-90°F.

Supplement to batter	Inoculated cake				Uninoculated cake			
	Mold growth on slice in Petri dish after days indicated							
	2	3	4	5	2	3	4	5
Control	−	+	+++	++++	−	+	+++	++++
Sorbic acid, 0.07%	−	−	++	++++	−	−	−	+
Sodium propionate, 0.28%	−	−	+	+++	−	−	+	+
	Mold growth on cake in humidity can after days indicated							
	1	2	3	4	2	3	4	5
Control	+	++++	++++	++++	+	+++	++++	++++
Sorbic acid, 0.07%	−	++	+++	++++	−	−	+	++
Sodium propionate, 0.28%	−	++	+++	++++	−	−	−	+

[1] Cakes varied in moisture content from 26.4 to 27.9% and in pH from 6.8 to 7.0.

this series the sorbic acid cakes were more resistant to mold spoilage. This was attributed in large measure to the limited value of the propionate as a fungistatic agent for cakes.

In Table 9 are presented results obtained in using the fungistatic agents to protect yellow pound cake produced on a commercial scale. In this study the mold inoculum varied from that employed in the earlier studies. One series of cakes was inoculated with a higher concentration of mold spores than that previously used, while another series of cakes was stored *without* deliberated inoculation. The findings obtained in this study show that sorbic acid was between 3 to 4 times as effective as propionate in protecting the yellow pound cake against mold spoilage. Here again, sorbic acid did not affect the aroma and taste of the cake and had no adverse effect on the volume, grain or texture.

There were suggestions in the studies with yellow pound cake that the fungistatic agents were both more effective in protecting cakes against spoilage due to adventitious mold contamination. This led to the design of an experiment to evaluate under practical operating and holding conditions the effectiveness of the two fungistatic agents in protecting cakes. For this study the marble pound cake produced on a commercial scale was selected as the test substrate. Sorbic acid concentration in both white and chocolate batters was increased making the ratio of sorbic acid to propionate 1:2. With sorbic acid it was possible to increase concentration without crossing the taste threshold.

Slices of marble loaf cake were prepared in accordance with regular production procedure. The slices, each measuring approximately 5 in. x 3 in. x ¾ in., were wrapped in moisture-proof heat-sealable cellophane on the mechanical equipment regularly used for this purpose. In these operations the cut surfaces were fortuitously contaminated with the mold spores present in the slicing and wrapping areas. Experience has shown that mold spores are always present in such areas despite elaborate sanitary measures. Ninety cake slices comprised each series for the evaluation of susceptibility to mold spoilage; the cakes in one series contained no added fungistatic agent, while those in the other two series were made with either sorbic acid or sodium propionate added as described in Table 10.

The wrapped cake slices were laid flat on top of a large table in an air-conditioned room. During the period of storage the temperature varied between 72-75°F.; relative humidity was maintained at about 50% with draft ventilation. For fear that the cakes might dry out during storage under these conditions, a large sheet of Saran film was placed over the slices in their cellophane wrappers and the periphery of the Saran film sealed by Scotch Tape to the table edges. The cakes were exposed to the subdued natural light of the room. At the conclusion of the test (17 days of storage), the cakes were also examined on the reverse surface that had rested on the table top. The results of this study are summarized in Table 10.

TABLE 10

Relative value of sorbic acid in moderate concentrations in protecting marble pound cakes, produced on a commercial scale, against spoilage due to fortuitous mold contamination

Fungistatic agent added	Added to batter				pH of cakes	
	White	Chocolate	White	Chocolate	White	Chocolate
	oz. per 460 lb.		Per cent			
Control	0.0	0.0	0.000	0.000	6.90	7.10
Sorbic acid	5.5	9.0	0.075	0.122	6.70	6.90
Sodium propionate	11.0	18.0	0.150	0.244	6.90	7.15

Mold growth	% cakes exhibiting mold growth on upper surface[2] after days indicated								% cakes moldy on reverse side[3]
	3	5	7	9	11	13	15	17	
On cake slice[1]									
Control	0	2	7	13	22	27	64	93	84
Sorbic acid	0	0	0	1	4	4	16	29	21
Sodium propionate	0	1	2	6	9	11	27	39	32
On white portion									
Control	0	2	4	6	11	13	49	84	72
Sorbic acid	0	0	0	0	2	2	14	28	20
Sodium propionate	0	1	2	6	8	8	22	34	27
On chocolate portion									
Control	0	0	3	10	16	19	49	79	58
Sorbic acid	0	0	0	1	2	2	7	18	12
Sodium propionate	0	0	0	0	1	3	12	20	16

[1] Ninety cake slices in individual cellophane wrappers constituted each series. The cake contained 28.8% moisture.

[2] The wrapped cake slices were laid flat on a table top in a room at 72-75°F., with one surface of the slice exposed throughout the holding period to the subdued natural light of the room.

[3] Only at the conclusion of the test (17 days of storage), were the cakes examined on the reverse side for mold spoilage.

The more important observations made and conclusions drawn in the course of this investigation of the resistance of commercially-produced cakes to mold spoilage are listed:

1. Marble pound cake slices not protected by a fungistatic agent are surprisingly susceptible to mold spoilage. Mold growth was noted in 2% of the slices within 5 days after baking and by 7 days had increased to 7%. At the end of 17 days fully 93% of the slices exhibited mold growth.

2. Propionate has a valuable protective function in protecting cakes against fortuitous mold contamination. Cakes were protected about 2 days longer under the conditions of test, 7 to 9 days before mold growth had progressed to a significant degree (2 to 6% of the slices moldy).

3. Sorbic acid in the concentrations employed increased still further the marketable period of the cake slices. Fully 9 to 13 days were required before mold growth was noted in 1 to 4% of the slices.

4. The superiority of sorbic acid over the propionate, the former used in one-half the concentration, is noted not only in the extension of shelf-life of the cake before mold spoilage occurs but also in the reduced incidence of mold spoilage at the conclusion of the test.

5. Mold growth on the reverse bottom surface of the cake slices represents growth independent of that on the upper surface. Examination of the reverse bottom surface of the cake slices confirmed the qualitative and quantitative superiority of sorbic acid over propionate in protecting in essence another series of cakes against mold spoilage.

6. Magnitude of mold growth parallels frequency. In the sorbic acid and propionate series, degree of mold contamination was far less than that noted with the control series.

7. The chocolate portion of the cake, subjected to fortuitous contamination, is no more susceptible to mold spoilage than the white, judging from the results obtained in evaluating the control series of cake slices. Indeed the chocolate portion of the cakes with the added fungistatic agents was more resistant to mold spoilage than the white, but this portion contained 64% more fungistatic agent than the white.

8. By comparing the effectiveness (in relationship to concentration) of sorbic acid in protecting the white portion of the cake with that of propionate in protecting the chocolate portion, it is seen that sorbic acid is somewhat more than 3 times as effective as the propionate. By comparing the effectiveness of sorbic acid in protecting the chocolate portion of the cake with that of the propionate in protecting the white portion, it is seen that sorbic acid will almost double shelf-life of the propionate-protected cake despite use of sorbic acid at a level of about 80% of that of the propionate.

9. Sorbic acid in the concentrations employed does not affect the aroma and taste of the cake and has no adverse effect on the volume, grain or texture.

The usual laboratory tests, involving deliberate inoculation of cake slices with mold spores and storage under conditions favorable to mold propagation, were also conducted on the same series of cakes described in Table 10. In confirmation of the previous laboratory studies, the fungistatic superiority of sorbic acid over the propionate was again demonstrated.

SUMMARY

The literature covering the use of sorbic acid as a fungistatic agent for protecting a variety of foods has been reviewed.

Sorbic acid is superior to calcium or sodium propionate or to sodium benzoate in protecting cakes against mold spoilage. It is approximately four times as effective as the propionate, i.e., one part of sorbic acid is as effective as about four parts of propionate when the latter is used at a high enough concentration to exhibit measurable fungistatic activity. When used at the same concentration of 0.20% (expressed on the batter weight basis), the extension of mold-free life of the cakes with added sorbic acid is about four times that of the cakes made with added propionate. Whereas the fungistatic activity of sorbic acid is increased with a lowering of the pH of the cake, the superiority of sorbic acid over the propionate is not due simply to differences in pH between cakes made with the fungistatic agents; sorbic acid is inherently four times as effective as propionic acid in protecting cakes against mold spoilage. The quantitative fungistatic superiority of sorbic acid over propionate established in laboratory studies has also been confirmed by tests conducted on cakes baked, sliced, and wrapped on a commercial scale.

Propionates can be used at only limited concentrations without imparting a flavor to the cakes. This restricts its use level to such low concentrations that the fungistasis attained is much less than desired. With sorbic acid used at the 0.1% level (expressed on a batter weight basis), a significantly greater degree of protection against mold spoilage is now obtained without alteration of cake flavor. There would seem to be little reason to increase sorbic acid concentration in cakes above the 0.20% level. There is no objection on the part of the U. S. Food and Drug Administration to the use of sorbic acid in concentrations up to 0.30% in place of propionates in cakes.

No measurable loss of sorbic acid occurs during cake manufacture.

LITERATURE CITED

1. Beneke, E. S., and Fabian, F. W. Sorbic acid as a fungistatic agent at different pH levels for molds isolated from strawberries and tomatoes. *Food Technol.*, 9, 486 (1955).
2. Borg, A. F., Etchells, J. E., and Bell, T. A. The influence of sorbic acid on microbial activity in commercial cucumber fermentations. *Bact. Proc., Abstracts*, 19 (1955).
3. Boyd, J. W., and Tarr, H. L. A. Inhibition of mold and yeast development in fish products. *Food Technol.*, 9, 411 (1955).
4. Bradshaw, W., Carbide and Carbon Chemicals Co. (Personal communications, 1955).
5. Costilow, R. N., Ferguson, W. E., and Ray, S. Sorbic acid as a selective agent in cucumber fermentations. I. Effect of sorbic acid on microorganisms associated with cucumber fermentations. *Bact. Proc., Abstracts*, 19 (1955).
6. Deuel, H. J., Jr., Alfin-Slater, R., Weil, C. S., and Smyth, H. F., Jr. Sorbic acid as a fungistatic agent for foods. I. Harmlessness of sorbic acid as a dietary component. *Food Research*, 19, 1 (1954).
7. Deuel, H. J., Jr., Calbert, C. E., Anisfeld, L., McKeehan, H., and Blunden, H. D. Sorbic acid as a fungistatic agent for foods. II. Metabolism of α,β-unsaturated fatty acids with emphasis on sorbic acid. *Food Research*, 19, 13 (1954).
8. Etchells, J. L., Bell, T. A., and Borg, A. F. The influence of sorbic acid on the growth of certain species of yeasts, molds, and bacteria. *Bact. Proc., Abstracts*, 19 (1955).

9. GOODING, C. M., MELNICK, D., LAWRENCE, R. L., AND LUCKMANN, F. H. Sorbic acid as a fungistatic agent for foods. IX. Physico-chemical considerations in using sorbic acid to protect foods. *Food Research*, 20, 639 (1955).
10. JONES, A. H., AND HARPER, G. S. A preliminary study of factors affecting the quality of pickles on the Canadian market. *Food Technol.*, 6, 304 (1952).
11. KIRK, J. K., TO GILBERT, A. M. Letter allowing the use of sorbic acid as a mold inhibitor in cakes. U. S. Food and Drug Administration, June 27, 1955.
12. LUCKMANN, F. H., AND MELNICK, D. Sorbic acid as a fungistatic agent for foods. X. Spectrophotometric determination of sorbic acid in foods in general. *Food Research*, 20, 649 (1955).
13. MELNICK, D., AND LUCKMANN, F. H. Sorbic acid as a fungistatic agent for foods. III. Spectrophotometric determination of sorbic acid in cheese and in cheese wrappers. *Food Research*, 19, 20 (1954).
14. MELNICK, D., AND LUCKMANN, F. H. Sorbic acid as a fungistatic agent for foods. IV. Migration of sorbic acid from wrapper into cheese. *Food Research*, 19, 28 (1954).
15. MELNICK, D., LUCKMANN, F. H., AND GOODING, C. M. Sorbic acid as a fugistatic agent for foods. V. Resistance of sorbic acid in cheese to oxidative deterioration. *Food Research*, 19, 33 (1954).
16. MELNICK, D., LUCKMANN, F. H., AND GOODING, C. M. Sorbic acid as a fugistatic agent for foods. VI. Metabolic degradation of sorbic acid in cheese by molds and the mechanism of mold inhibition. *Food Research*, 19, 44 (1954).
17. PATRICK, R., AND ATKINS, C. D. Effectiveness of sorbic acid as a preservative for tangerine sherbert base. *Proc. Florida State Horticultural Soc.*, 67, 194 (1954).
18. PHILLIPS, G. F., AND MUNDT, J. O. Sorbic acid as inhibitor of scum yeasts in cucumber fermentations. *Food Technol.*, 4, 291 (1950).
19. PULS, D. D., LINDGREN, L. F., AND COSGROVE, F. P. Sorbic acid as a fungistatic agent for certain pharmaceutical preparations. *J. Am. Pharm. Assoc., Sci. Ed.*, 44, 85 (1955).
20. Research Laboratories, The Best Foods, Inc., Sorbic acid studies (1938-1955); data to be published.
21. SHENEMAN, J. M., AND COSTILOW, R. N. Sorbic acid as a preservative for sweet cucumber pickles. *Applied Microbiol.*, 3, 186 (1955).
22. SMITH, D. P., AND ROLLIN, N. J. Sorbic acid as a fungistatic agent for foods. VII. Effectiveness of sorbic acid in protecting cheese. *Food Research*, 19, 59 (1954).
23. SMITH, D. P., AND ROLLIN, N. J. Sorbic acid as a fungistatic agent for foods. VIII. Need and efficacy in protecting packaged cheese. *Food Technol.*, 8, 133 (1954).
24. STEPHENS, M. R., TO GILBERT, A. M. Letter on the harmlessness of sorbic acid as a food component. U. S. Food and Drug Administration, May 7, 1953.

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